PHYSIOLOGICAL AND BIOCHEMICAL STUDIES ON VERNALIZATION AND COLD HARDINESS OF WHEAT

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PHYSIOLOGICAL AND BIOCHEMICAL STUDIES ON VERNALIZATION AND COLD HARDINESS OF WHEAT

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The undersigned hereby certify that they have read, and recommend to the School of Graduate Studies for acceptance, a thesis entitled "Physiological and Biochemical Studies on Vernalization and Cold Hardiness of Wheat," submitted by James Banting, B.S.A., M.Sc., in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

ABSTRACT

An attempt has been made to establish a reasonably quick and accurate laboratory method for rating hardiness of selected winter wheat varieties.

One phase of the work involved a study of selected varieties vernalized for periods ranging from zero to ninety days and subsequently grown to heading in greenhouse beds. This was intended to reveal a possible correlation between completeness of vernalization (determined by the number of days from planting to heading) and the known cold-hardiness rating of the selected varieties, as previously determined by winter-survival tests. The resulting data have shown no correlation between the factors studied.

The second phase included comprehensive tests under controlled hardening and freezing conditions in the germinative and two to four-leaf stages of growth, with and without soil, light, and various chemical treatments. The results appeared to justify the following conclusions:

1. Unhardened material in either the germination or the two to four-leaf stage of growth, when tested by both slow and quick-freezing techniques, is an unreliable index of cold hardiness in comparison with the known winter-survival rating.

2. Germinating seedlings, vernalized for periods ranging up to 80 days, gradually increased in cold resistance up to the sixtieth day, which corresponded to the point of complete vernalization. Beyond this point there was a marked decline in cold tolerance.

3. Subjection of free, intact wheat seedlings in the germinative stage to constant low temperatures for periods varying from two to fourteen days, prior to freezing, indicated: (a) that it was impracticable to assess freezing injury in such completely exposed material; and (b) that freezing tests on germinating seedlings grown in vermiculite and hardened for periods of seven and fourteen days in continuous light gave a fairly satisfactory index of hardiness, while shorter hardening periods (up to three days) were less reliable.

4. Longer-term hardening treatments at controlled low temperatures and continuous illumination, for periods of three to six weeks, applied during the two to three-leaf stage to seedlings grown in pots and finally subjected to slow-freezing techniques, gave a satisfactory hardiness rating in comparison to winter-survival data.

5. Pre- and post-germinative application of various chemicals - in particular, Dalapon (sodium 2,2-dichloropropionate) and maleic hydrazide - gave erratic results, which failed to indicate any consistent increase in cold resistance attributable to the applied chemicals.

Biochemical analysis of the material, which had been subjected to the different hardening conditions, included analysis for several nitrogen fractions, as well as sugar and starch determinations on a portion of the material. Some of the results obtained do not agree with data reported by other workers for various plant materials. The implications of the observed results with vernalized material have been discussed with reference to previous work, and an alternative explanation offered.

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INTRODUCTION

Since the range of winter wheat is largely determined by the plants' ability to resist frost, the problem of testing for cold resistance assumes major importance in the development of hardier winter wheat types. While the breeding of hardy winter wheats is a matter of great economic importance throughout the world, it is also an unfortunate fact that winter-hardy forms are scarce among the world's wheat varieties. This knowledge, and the knowledge that our dwindling resources of arable land lie generally northward, has placed the plant breeder in a challenging situation.

Under natural conditions, the varieties or strains may be grown for several seasons before an adequate "test" winter reveals the desirable hardier types. The preferred degree of injury, which eliminates the tender, and yet shows differences within the hardier strains, is difficult to obtain in the field. Thus the methods of testing for hardiness within winter wheat varieties are both time-consuming and costly.

The problem appears to be solved satisfactorily, under generally milder winter conditions than prevail in Western Canada, by means of controlled freezing tests on field-hardened material. With increased duration and severity of winter, however, the utilization of field-hardened material becomes increasingly difficult, and even impossible, suggest-

ing the use of both controlled hardening and freezing tests as a possible solution to the problem.

The major objective of the studies reported here was to establish a reasonably quick and accurate laboratory method for the determination of the relative hardiness rating of winter wheat varieties. In order to increase the scope of the studies, the work was divided into the following three groups:

I. A study of the effects of varying periods of vernalization on subsequent development of selected wheat varieties, as determined by the number of days required from planting to 50 and 100 per cent heading. This was intended to permit a search for a possible relationship between completeness of vernalization and the known cold-hardiness rating of the varieties, as previously determined from winter survival tests. Since it is believed that the hardier varieties usually require long periods for complete vernalization, and that this is a desirable characteristic, which also differs between varieties, it was hoped that some correlation might exist between the period required for vernalization and the inherent cold resistance of a variety.

II. Controlled freezing tests on unhardened, vernalized, short and long-term hardened, and chemically-treated material. These tests were carried out in the germinative and 2 to 4-leaf stages of growth, with and without soil or vermiculite,

in the presence or absence of light, and under the effects of various growth substances.

III. Biochemical analyses on material which had been subjected to different hardening treatments in the germinative and seedling stages of growth. The major part of this phase of the work included determinations of various nitrogen fractions. A limited number of sugar and starch determinations were also included.

LITERATURE REVIEW

Although the original literature pertaining to vernalization appeared in Russian in 1930, it did not become generally available to the English-speaking world until 1933, when the Imperial Bureaux of Plant Genetics published a paper by Whyte and Hudson (69), entitled, "Vernalization or Lysenko's Method for the Pre-treatment of Seed," which dealt with the practical details of vernalization of individual plants. The following year, the Bureaux presented a paper, in collaboration with Maximov (38), on the theoretical aspect of vernalization, which outlined and discussed briefly Lysenko's conceptions. In this publication, vernalization was defined as "a practical agricultural method of accelerating the development of plants." Presumably, the usefulness of Lysenko's technique in winter cereals was based on the restriction of growth in the germinative stage by limitation of the moisture supply during the low-temperature treatment, thus permitting the treated grains to be sown in the seed drill. As Whyte (68) has indicated, Tolmacev had developed a similar method at this time with practically identical results.

Details of Lysenko's conceptions and his controversial theory of phasic development, which followed his work on vernalization, are given by Maximov (38), Murneek and Whyte et al (45), and Whyte (68), and will not be discussed here.

Suffice it to say that the trend of Russian developmental physiology was greatly influenced by Lysenko's theoretical principles, which at the same time opened an extensive and fertile field of research to physiologists in general.

It should be pointed out that in the years up to 1918 the pioneer researches of Klebs, who emphasized the possibility of controlling reproduction by external influences, contained almost all the main facts elicited by Russian theories of vernalization and photoperiodism. According to Maximov (38), Lysenko believed that the completion of every stage of development in a plant signified in itself that a definite qualitative and irreversible change had taken place. He also believed that vernalized seeds could be stored for considerable periods without loss of their vernalized quality, provided the tissues that had acquired the vernalized condition were not damaged in any way. In addition, he opposed the hormone theory of flower development and did not think that the state of vernalization could spread from one part of a plant to another. These statements have since been disproven in part or in whole. The phenomenon of devernalization has been reported in winter rye by Gregory and Purvis (19), Purvis and Gregory (52), Stout (61) with beet, and others. Purvis and Gregory (52) have shown that "the degree of devernalization by exposure to subsequent high temperature varies with the initial duration of vernalization and becomes progressively less as vernalization proceeds." After heat devernalization,

they succeeded in revernalizing the grain by further exposure to low temperature, and thus established the reversibility of vernalization in either direction. They also found that after 12 weeks' vernalization no devernalization occurred in winter rye from subsequent heat treatments, which indicated that the vernalized condition became progressively stabilized with increasing periods of low temperature. Further studies by Friend and Gregory (16) revealed another effect of temperature on partially vernalized grain in which longer periods of heat treatment, up to six weeks at 20° C. and 25° C., respectively, were employed. Their data indicated that such prolonged heating of partially vernalized winter rye led to an intensification of vernalization, as shown by the acceleration in time to flowering. Such was not the case in spring or fully-vernalized winter rye.

The loss of vernalization by drying vernalized grain for periods longer than six weeks has also been demonstrated by Gregory and Purvis (20) in winter rye, although lack of success attended similar experiments in winter wheat. However, Lojkin (34) has reported a partial or complete loss of vernalization in fully vernalized seed kept air-dried for four weeks at 1° C. and 15° C. Similar results have been recorded by certain Russian workers. Various investigators (20, 34) have indicated the difficulty of maintaining the recommended moisture level of the grain in the Russian technique of vernalization, and have shown that even very slight

drying completely inhibits the process. Preliminary experiments on vernalization of seven winter and one spring variety of wheat, by the writer, using the above technique, corroborate these results. Failure to obtain heading of the winter wheat varieties in the field, when vernalized for periods ranging up to 50 days, was attributed to slight drying-out of the grain during low-temperature treatment. In discussing similar results by Lojkin, Gregory and Purvis (20) were of the opinion that the vernalization achieved in the earlier stages of germination was later completely annulled by drying.

Murneek, Whyte et al (45) attach great economic significance to the possibility of vernalization before seed-ripening in the field, and cite the work of Kostjucenko and Zarubailo in Russia and Gregory and Purvis in England. The Russian workers were concerned with natural vernalization in heads of winter wheat during ripening. Gregory and Purvis (20) applied artificial chilling to winter rye heads varying in maturity from five to thirty-five days after anthesis. They showed that low-temperature vernalization was effective from the earliest stage of the embryo and ceased as it became dormant. However, similar experiments carried out on four Russian winter wheat varieties failed to indicate successful ear vernalization.

The relation between the effect of vernalization of ripening grain during development and the loss of this effect as demonstrated in dried-down vernalized grain is dif-

ficult to interpret. As Gregory and Purvis (20) state, "If the drying-down of the vernalized mature embryo leads to disappearance of vernalization, it might be expected that the embryos exposed to low temperature during development would later lose this effect while dormant. Manifestly this is not so in the case of winter rye, though the failure of ear vernalization in wheat might be interpreted in this way. At present there is no clear solution of these apparently contradictory results."

As stated previously, Lysenko did not think that the state of vernalization could spread from one part of a plant to another. According to Gregory and Purvis (20), and Gregory (18), the effect can spread. By removal of the main axis of vernalized winter rye plants as soon as possible after germination, and of the tillers as they appeared over a period of time, they found that the vernalized state was still transmitted to the later-developing tillers, which produced heads much earlier than did the main axis of unvernalized and unmutilated plants.

Gassner's work on temperature in the early stages of plant growth, just after germination, is reported in some detail by Whyte (68). In addition to the belief that low temperature induced increased winter-hardiness in winter rye plants, Gassner believed that their subsequent flowering was dependent to a large extent on their passing through a period

of low temperature either during germination or at some stage subsequent to germination. He also believed that "winter-hardiness and the cold requirement, so important for flowering, were correlatively connected."

Rudorf, another German worker, studied the relation between vernalization and photoperiods and resistance to cold, details of which are reported by Murneek, Whyte et al (45). According to Rudorf (54), one of the indirect practical advantages of vernalization for breeding is its use in winter cereals for testing winter-hardiness. He mentions that different varieties require different exposures to cold in the grain or seedling stages before they will come into ear when sown in the spring, and states, "As a rule the varieties that require the shortest exposures to cold are also the least winter-hardy." Further details of this work will be discussed later.

According to Maximov (38), Lysenko considered that the degree of hibernalization (strong preservation of the winter state) was indicated by the duration of low-temperature exposure required for vernalization. He believed that the vernalization period varied from 15 to 60 days or more, and respective weak, medium and strong winter forms could be distinguished. Builina (4), in his studies on winter-hardiness in wheat, published results which more or less confirmed Lysenko's conception, although some exceptions were recorded.

He found that for fairly complete earing the wheats of low winter-hardiness required 46 days' chilling; wheats of average winter-hardiness and some of the winter-hardy varieties required at least 58 days' chilling; while one winter-hardy type required at least 70 days' chilling. After testing some 28 or more wheat varieties of varying winter-hardiness, over a period of three years, his tentative conclusions were as follows:

(a) All varieties eared more completely and the flowering period was shortened according as the duration was prolonged to an optimum figure specific for each variety.

(b) As a rule, the optimum duration of chilling is proportional to the degree of winter-hardiness of the variety, although some exceptions were recorded.

(c) At the present time, the following durations of chilling can be suggested: low winter-hardiness - 45 to 55 days; average winter-hardiness - 55 to 60 days; winter-hardy - 60 to 65 days.

As reported by Levitt (32), Saltykovskij and Saprygina point out that "the frost resistance of winter cereals does not depend entirely on the length of the yarovisation period. Thus, varieties having the same length of period may differ greatly in frost resistance, while those with unequal periods may be equally resistant. Yet there are no hardy

varieties with a short first stage, so it is still one of the principal features of hardiness." In a later study of wheat and wheatgrass hybrids, the senior author reported that cold resistance was higher the longer the developmental phases.

Various Russian workers (32) have reported a correlation in winter cereals between hardiness and the vegetative period of the plants, as measured from germination to heading time. In their study of growth habit of winter wheat, Quisenberry and Bayles (53) found that the degree of winter habit was not closely related to time of heading from fall seeding or to winter-hardiness. In all cases the early varieties were less hardy than most of the late ones, but a few of the late varieties were no more hardy than early maturing ones.

McKinney and Sando (41) studied the influence of temperature and photoperiodism on earliness and seasonal growth habit in wheat. They showed that, during the vernalization period, temperatures from 37° to 44° F. (2.8° to 6.6° C.) were more favorable than lower temperatures for stimulating earliness in the winter varieties tested. At the same time, the low temperatures retarded earliness in the spring wheat varieties. They also studied the effect of temperature and photoperiod on the number of days required from the end of various vernalization periods to heading. They found that more varieties failed to head at 70° to 90° F. in the greenhouse under natural daylight than at the lower outdoor temperatures pre-

vailing with a natural day, while the number of failures were least with uninterrupted light at the outdoor temperatures. They concluded that the optimum vernalization period would be determined not only by varietal characteristics but also by the temperature and light conditions obtaining during the subsequent growing period. In a further test with winter wheat, they succeeded in obtaining plants, which headed earlier and yielded more than those from chilled seed, by using various combinations of temperature and photoperiod, ranging from 50° to 75° F. in the former, and from 8 to 18 hours in the latter. Results from a more comprehensive test, where several winter wheat varieties were grown under still more variable conditions, showed wide differences between varieties in their earliness reactions when the plants received light daily during the period of low temperature. They also observed that certain varieties reversed their relative order of heading due to the changing influence of temperature and photoperiod in the different treatments, and that this also occurred in these varieties in the field at different stations. They noted, further, that the phenomenon has been observed in other varieties by several workers in different parts of the world. In a recent study of the responses of spring wheat varieties to day-length at different temperatures, Gries et al (21) reported a reversal in earliness due to differential response to temperature. Vlitos and Meudt (67), working with spinach (var. Nobel), found the critical period necessary for flower-

ing in this variety was lowered from 14 to 8 hours as a result of vernalization.

McKinney and Sando (41, 42) state that "earliness and lateness of sexual reproduction appear to depend on the interrelation of several characters, as, the number of internodes per culm, the growth rate of the internodes, the duration of the elongation of the internodes, and environmental-response characters which influence the expression of these several characters." They also note that temperatures and photoperiods favouring earliness in spring and winter wheats favour the formation of a reduced number of internodes and leaves by each tiller. As Murneek and Whyte et al (45) point out, in relating environmental response to number of internodes, McKinney and Sando forecasted the number-of-leaves interpretation put forward by Gregory and Purvis.

Further details on vernalization and/or photoperiodism may be found in reviews by Maximov (38), McKinney (40), Gregory (18), Murneek and Whyte et al (45), Whyte (68), and Lang (30). A recent review on the physiology of flowering, by Liverman (33), does not deal with the new advances in vernalization.

The next section of this review is concerned mainly with conditions relating to, and factors affecting, controlled low-temperature hardening and freezing experiments. Accurate measures of hardiness under field conditions are usually dif-

ficult due to variations in soil, in weather conditions, and often in plants. On the other hand, the temperatures, light intensities and photoperiods or their combinations, best suited for hardening in wheat are not known with any certainty. According to Martin (37), wheat plants without snow protection have survived temperatures of -25° C. or lower. Maximov (39) quotes a survival temperature of -15° to -20° C. for some winter cereals even in snowless winters. Worzella and Cutler (71) believe that temperatures of 0° to +5° F. (-17.7° C. to -15° C.) may be lethal to well-hardened wheat seedlings. It is evident from these statements that the hardier winter wheat varieties may withstand a considerable degree of cold under suitable hardening conditions. To the best of this writer's knowledge, the only claim to attainment of such a degree of hardening in winter wheats under laboratory conditions is that made by Tumanov and his associates, as reported by Whyte (68). Accordingly, the following two sections on (a) the nature of hardening and (b) the relation between phasic development and winter-hardiness, as Tumanov is concerned with them, will be dealt with in their respective order.

Tumanov recognizes that a high degree of frost resistance is not a property which is always inherent in a given plant. In the development of this quality, he notes that plants must undergo certain internal readjustments -- processes which are not yet fully understood and which are known under the general name of hardening. Tumanov and his

associates claim the establishment of two ecologically and physiologically distinct phases in the hardening of plants, this being the main distinction between Russian research as compared to Western European and American research where, according to Tumanov, only the first of the two phases has been applied.

The acquisition of the first phase of hardening occurs in light with a temperature range of 0° to 6° C., the latter temperature apparently limiting too extensive growth, and in so doing aids the promotion of a large accumulation of sugars in the plant. Under laboratory conditions, this state may be fully acquired in 5 days or less, the first resistance acquired with it being limited, with lethal temperatures for winter wheats varying from -7° or -8° C. to -10° or -12° C. Tumanov also considers that hardening would be possible at temperatures alternating from 10° to 15° C. during the day to 0° C. at night, in which case assimilation and storage processes would be stimulated during the day and growth processes arrested at night. Under these conditions, however, plants exhibit a marked reduction in their ability to achieve the second phase of hardening, the failure of which Tumanov believes may be due to too rapid growth during daylight.

The second phase may be initiated and proceed only when and if the first phase is completed. This phase is acquired only under much lower temperatures, in the vicinity of -2° to -5° C., and is presumed to be based upon different

physiological processes. The phase is similar in its effects to intensive wilting, since under the specified conditions a dehydration process occurs due to the freezing of water in the tissues. The second phase progresses more rapidly than the first, although excessive moisture in the environment of the plant may cause a retarding effect.

As an example of the marked increase in frost resistance due to this second phase of hardening, Whyte (68) records Tumanov's example in Lutescens wheat, the percentage survival being increased from 17 at -13° C. after the first phase to 96 at -17° C. after the second phase. As noted above, the second phase follows upon completion of the first, at which time, Tumanov states, dehydration of tissues and low temperature may become effective only in the presence of the protective substances of the first phase at a definite developmental phase. Whyte (68) states, "It is not known on what basis Tumanov assumes there are two clear-cut phases; hardening appears to be more generally recognized by other physiologists as a continuous process."

Numerous Russian investigators have claimed that frost resistance is connected with phasic development. Experimental data, concerned mainly with winter cereals, have shown that frost resistance is generally lower in plants from vernalized seeds, often markedly so. Such results have an important bearing on the conditions obtaining in the field

where vernalization may be fully or partially completed during the winter. Some investigators have shown that frost resistance falls rapidly in vernalized plants when grown under conditions of higher temperatures and long days; they believe, however, that frost resistance is maintained until such conditions prevail. Others have stated that decreased frost resistance may be noted when vernalized plants are grown under conditions which do not favour the photo-phase (high temperatures and long days). On the other hand, according to Whyte (68), Tumanov considered that completion of vernalization alone did not necessarily mean that frost resistance was markedly reduced, provided the conditions for subsequent growth were not present. However, he found that vernalized plants showed no increase in their frost resistance when exposed to the second phase of hardening under the appropriate conditions, although subjection to this phase should produce increased hardiness.

As mentioned above, Builina (4) reported a close relationship between length of vernalization period and frost resistance - the hardier the variety the longer the required period for complete vernalization, although he did state that there were exceptions. Differences in frost resistance between varieties with similar vernalization periods have been reported by other Russian workers, which forced Tumanov to conclude that Builina's claims were incorrect. This did not imply, however, that the length of the vernalization period

was unimportant, as obviously is not the case.

Further results of Rudorf's work are reported by Murneek and Whyte et al (45) and Whyte (68). Rudorf chilled winter cereals for periods ranging from 20 to 60 days at 1° to 3° C., and grew them subsequently either in an 8-hour or a 16-hour day. Without exception, cold resistance in all varieties was reduced through the long-day treatment, even after chilling periods of 40 and 60 days. Other results with winter, dual-purpose and summer forms of wheat and barley, which had been chilled for periods ranging from 20 to 60 days at 3° to 5° C., showed that frost resistance was largely determined by the condition of the plants in the dormant period before completion of vernalization. Cold resistance was gradually decreased as the degree of vernalization was increased. With partial completion of vernalization, hardening capacity was lowered to a degree which was in proportion to the length of the treatment. However, Rudorf noted a reversal in the reaction of certain varieties after 60 days' chilling; i.e., increasing cold resistance, which no doubt influenced the following considerations (which he suggested should be applied in future work): (a) isolation of varieties requiring a long period of vernalization with maximum cold resistance, and (b) the discovery of varieties which would still exhibit great hardening capacity after the low-temperature phase (vernalization period) was completed.

One of the earlier investigations on this continent on the measurement of cold resistance of winter wheats by artificially-produced low temperatures was carried out by Hill and Salmon (25). They found the method to be a very promising one for studying the relative hardiness of different varieties, and stressed the importance of the degree of hardening in obtaining the proper hardiness relationship between varieties. Previous studies by Newton (46) had indicated that the hardening process continued long after the advent of freezing weather, and thus the time interval was important in the development of maximum hardiness by certain varieties. Up to this time, numerous attempts had been made to determine the correlation of various physical, chemical, physiological and morphological characters with known frost hardiness. Martin (37) concluded, after such a study, that the only possible alternative to careful field study, in obtaining accurate results on hardiness in the laboratory, was perhaps a method of controlled freezing.

Since the inception of refrigeration techniques, numerous investigators - including Peltier and Tysdal (51), Anderson and Kiesselbach (2), Suneson and Peltier (62), Worzella and Cutler (71), and others - have reported a close agreement between hardiness rating of winter wheat varieties as found in the laboratory under controlled freezing conditions and winter survival data obtained from field studies. The experiments generally utilized field-hardened material, and

it was suggested by Anderson and Kiesselbach (2) that controlled hardening was unnecessary in such tests. However, they did note a few intervarietal changes in ranking, as well as striking fluctuations in the relative degree of survival. Suneson and Peltier (62) also found that considerable variation in the relative hardiness relations of varieties accompanied seasonal and environmental variations under field conditions. Laude (31) reported changes in the relative ranking of winter wheat varieties in the transitional period from dormancy to active growth in the spring. He observed that the dehardening process was relatively slow in some of the more tender varieties during this period, while the generally hardier types were not necessarily well-adapted to sharply varying temperatures at this time. Dexter (15) confirmed Laude's findings in his studies of the effects of warm weather upon the hardened condition of winter wheat plants. On the other hand, although Worzella and Cutler (71) recorded wide varietal variations in relative cold resistance of winter wheats, they found no change in varietal rank or reversal of varietal hardiness during the winter period. Their hardiest variety possessed the highest level of cold resistance, accumulated the greatest degree of hardiness during cold periods, acquired hardiness earlier in the fall and maintained this condition later in the spring.

In the writer's opinion, the foregoing results serve to illustrate the need for more detailed information with re-

spect to the factors affecting controlled hardening of winter wheats in the laboratory. Until a high degree of hardening is attained under controlled conditions, the study and evaluation of hitherto uncontrolled environmental changes is difficult and often inconclusive.

Suneson and Peltier (63) studied the effect of stage of development upon the cold resistance of winter wheat seedlings, grown in the greenhouse for various periods prior to controlled hardening and freezing. They found the youngest plants, which were hardened 4 days after emergence, were the most cold-resistant. Peltier and Kiesselbach (50) reported similar findings in spring cereals under controlled conditions. In addition to the one-leaf stage, they found that seedlings with four leaves or more showed increased cold tolerance compared to the two and three-leaf stages. Whether comparisons are valid or not, under the stated conditions, is debatable. Working with field-hardened varieties of winter wheat, Worzella and Cutler (71) found that germinating seedlings in the one-leaf stage were quite susceptible to cold. Beyond this stage, cold resistance increased through the two to four-leaf stage, reaching a maximum at the five to fifteen-leaf stage. More advanced seedlings, ten to twelve inches in height, showed a considerable decrease in cold tolerance. Worzella (70) obtained similar results in earlier experiments with seedlings ranging in age from fourteen to forty-six days, which were grown in the greenhouse during the period, December

to March, and subjected to fifteen hours' hardening at 34° F. under artificial light. He concluded that up to the age of thirty-two days the seedlings were less cold-resistant than older plants. Beyond this stage, the plants showed approximately the same percentage survival. Martin (36) studied the cold resistance at various stages of growth of certain commonly-fall-sown spring wheat varieties. He noted that differentiation of varieties, with respect to cold resistance, was difficult in the more advanced stages of development, especially where varietal differences were small. Considering the wide variations in the methods used by these investigators to promote the hardening process, the lack of agreement concerning stage of growth and the development of maximum cold resistance is not surprising.

As mentioned previously, detailed information on the temperatures and photoperiod best suited for hardening in winter wheats is meagre, especially under controlled conditions. In addition, the time required for the development of maximum hardening is not known. Previous investigators have been concerned mainly with developing a degree of hardening which would show wide varietal differences in freezing injury and not the ultimate in cold resistance.

Dexter (12) studied the effect of darkness, and different periods of light, as well as the influence of light with and without carbon dioxide, on the hardening of alfalfa,

wheat, cabbage and tomato plants. He also employed several constant temperatures and various combinations of alternating temperatures, both with and without carbon dioxide. The more succulent greenhouse-grown wheat plants did not harden in the dark at 0° C., but hardened well when illuminated, provided carbon dioxide was not removed from the air. Winter wheat plants, which had been grown in the field for five weeks during the autumn period, hardened markedly in the dark at temperatures near 0° C. Presumably, the effective hardening in the latter case was due to more abundant storage of organic food. Alternating temperatures in either light or darkness were not found to be particularly favorable to hardening. Similar results have been reported by Peltier and Kiesselbach (50) and Suneson and Peltier (63). The latter workers, however, found the order of hardiness of two varieties under constant low temperatures to be opposite to that shown under alternating temperatures during hardening, which suggested the importance in hardening of factors other than low temperature. Dexter also found that short periods at higher temperatures, with or without light, affected the hardening behaviour of winter wheats more adversely than that of alfalfa, cabbage or tomato plants. Long-day treatments at higher temperatures, which promoted elongation of foliar parts, were antagonistic to hardening in all cases.

Plants exposed to long-day illumination at 0° C. hardened more fully than with shorter periods of light, there

being no indication of extended growth in either case. In addition, winter wheat plants grown under short days in the greenhouse at 60° F. hardened more rapidly and more completely in the cold chamber than similar plants grown under long-day treatments prior to either a long or a short-day hardening treatment. The results of his experiments, in Dexter's opinion, pointed to similar conclusions; namely, "that hardening of plants is favoured by conditions which tend toward the accumulation or conservation of carbohydrates and other reserve foods - that is, which further photosynthesis and lessen respiration and extension of vegetative parts."

Dexter's (12) results did not appear to agree with Harvey's suggestion that hardiness in plants is a cold shock response. Harvey (22) found that the effect of exposures of as little as one to four hours per day, at 0° C., overbalanced the effect of longer exposures, 23 to 20 hours per day, at 10° and 20° C. Comparisons are difficult in this case, however, as Harvey was studying cabbage plants under continuous illumination. Dexter showed that the most efficient hardening treatment in alfalfa, winter wheat, and cabbage, was continuous light with continuous cold.

Tysdal (65) studied the influence of length of day, light intensity, and wave-length of light, as well as the interrelation between the influence of light intensity and day-length, on the hardening process in alfalfa under greenhouse

conditions. With the partial exception of the work of Dexter and his associates, similar details are lacking in studies with winter wheats, although exceptions to the hardening behaviour of alfalfa have been noted in winter wheats by Dexter (12, 14), Suneson and Peltier (63), Peltier and Kiesselbach (50), and others. Tysdal (65) found that length of day influenced the hardening process in alfalfa considerably. Within a temperature range of 10° to 12° C., the hardier varieties showed a marked response to a short-day length of 7 hours, while at 0° or 20° C. very little effect was evident. The hardiest variety responded the most to short day under the stated conditions. In addition to day-length, light intensity was found to be important only below a minimum which limited normal growth. At the range of optimum activity, Tysdal found that light appeared to influence hardening almost as much as temperature. The hardening behaviour of winter wheats, however, under similar circumstances is not well-defined.

Worzella (70), working with winter wheats, and more recently Amirshahi and Patterson (1), working with oats, have employed short periods of hardening (15 and 24 hours, respectively, at 34° F. with light) as a means of differentiating between varieties or groups, originally selected on the basis of a wide range in winter-hardiness. Whether such limited periods of chilling will distinguish small differences in cold resistance between varieties in their normal winter-hardened state, and in the correct hardiness relationship at all times,

remains to be seen. As noted above, reversals in the order of relative hardiness between varieties have been reported under both controlled and field-hardened conditions (50, 62). In view of these inconsistent results, the usefulness of very short periods of hardening may be limited.

Various investigators have studied the seasonal rise and fall of cold tolerance in winter wheats, including Dexter (13), Laude (31), Worzella and Cutler (71), and others. Tumanov, as reported by Meyer (43), found that winter wheat plants pass out of the condition of hardiness much more rapidly than they gain it. He showed that while a short exposure (24 hr.) to a temperature of 5° C. markedly increased their resistance, maximum resistance developed only with longer periods of two to three weeks. Dexter concluded "that the maintenance of the hardened condition in winter wheat plants was dependent upon environmental conditions which favour the conservation of organic food reserves; that is, which depress respiration and top growth and favour dormancy with continued periods of photosynthesis." With respect to respiration, Newton and Anderson (48) found that at 0° C. or lower respiratory activity was inversely related to winter-hardiness, which explained why the hardier winter wheat varieties maintained their sugar reserves better than non-hardy varieties during the winter.

A matter of importance in studies of this kind concerns the methods of freezing and thawing employed. In the

past 20 years, Scarth and his associates (chiefly Levitt and Siminovitch) have carried out extensive cell physiological studies of frost resistance with living material. Details of this work are summarized by Scarth (56). Siminovitch and Scarth (60) found that intracellular freezing, which is nearly always fatal, is facilitated by a rapid drop in temperature, its occurrence being less frequent in hardy tissues. According to Scarth (56), intracellular freezing may also occur on the breakdown of supercooling, although supercooling in itself is non-injurious. On the other hand, extracellular freezing, the only type which is compatible with survival of any great degree of frost, is induced through slow cooling and is fatal to unhardy cells at sufficiently low temperatures. According to Martin (37), winter wheat plants in an unhardened state will survive a temperature of -5° C. for 24 hours, while Newton and Anderson (48) state, "that -7° C. is the temperature at which absolute killing of unhardened greenhouse tissue occurs."

In addition to rate of freezing, the time factor serves to complicate the issue. Scarth notes that there is considerable evidence to show that death due to freezing is rapid at the critical temperature, although very slow injury may occur above this point. He also reports Levitt's findings, which showed that the duration of exposure to freezing temperatures had no distinct effect for short periods, but became more pronounced with a considerable increase in time.

Various workers, including Anderson and Kiesselbach (2), Worzella (70), and others, studied the effects of slow and rapid thawing on the percentage recovery of winter wheat plants after controlled freezing. The investigators appeared about equally divided as to the merits of each method, and in certain studies there was no significant difference. Scarth (56) and Levitt (32) seem to favour the view that if injury is produced by rapid thawing the temperature rise must be large, and when slow thawing is employed the rise in temperature should be gradual enough to permit diffusion of water molecules from ice to cell sap while the temperature is still below the melting point of ice. Under these circumstances, there would be no flooding of intercellular spaces with water at any stage.

There seems to be little agreement among the various investigators as to the critical portion of the winter wheat plant which may determine ultimate winter survival. Martin (37) believes the crown to be the most hardy portion of the wheat plant above the soil surface. He considers the young leaves to be more hardy than old leaves, and the bases to be more hardy than the tips. In their studies of respiration of winter wheat plants, Newton and Anderson (48) claim that the removal of the roots seems justifiable, since the leaves are the organs which determine winter survival. They are in agreement with Martin with respect to the leaves, but note that if all leaves are killed to the base the plant fails to survive. They believe that, while the roots and crown buds are the critical points in legumes such as clover and alfalfa,

this is not the case in cereals.

Dexter et al (11) developed a method for determining hardiness in plants in which they froze root samples of uniform weight, the inference being that these were the hardiest portions of the plant. In later studies, Dexter (13) defoliated field-hardened plants, and in so doing stimulated further foliar development. Almost four weeks later, following a period of cold weather, these plants were severely injured and were withered back to short stumps at the crown. Later development showed that while the plants were winter-injured they were not winter-killed. Suneson and Peltier (62) observed that field-hardened winter wheat plants, which had survived a severe freezing in the laboratory, consistently formed a new set of roots from the base of the crown. They found that plant development under these circumstances was very closely related to the rate of development of new roots, and reported no expansion or activity of the old roots. They concluded that wheat roots appear to be less hardy under certain exposures than the crown, or even the leaves, and that ability to regenerate a new root system is, in some instances, a factor in survival. Similar observations are reported by Janssen (28). He concluded that in the spring the new roots originate from the crown of the plant and not from the continuation of the old root growth, as is commonly assumed.

The effect of soil moisture upon the hardening process in plants has been studied by Klages (29), Martin (37), Janssen (28), Tysdal (65), and others. In general, it was found that low soil moisture favoured increased hardiness, while high soil moisture tended to reduce hardening. Numerous investigators have shown that cold resistance can be increased in winter wheats by subjecting them to various degrees of wilting. Scarth (55) has pointed out that drought-hardening in plants involves the same physiological changes as cold-hardening, and thus resistance to cold is associated with endurance of desiccation or dehydration. This principle has been utilized in cellular tests by Siminovitch and Briggs (58) to determine the degree of frost-hardiness in plants. In place of actual freezing tests, the degree of dehydration injury by plasmolysis has been used as a measure of freezing injury.

A point to remember with respect to soil moisture differences in freezing tests is the more rapid temperature drop in dry, as compared to wet soil, the slower rate in the latter being due to the greater specific heat of water as compared to soil. This indicates the importance of recording soil temperatures during freezing in comparative tests of this kind. Various investigators have taken the precaution to equalize the soil moisture prior to freezing to overcome these discrepancies.

The effect of synthetic growth regulators on frost resistance in plants is a relatively new and unexplored field. Recent studies by Corns (8, 10), Corns and Schwerdtfeger (9), and Miller and Corns (44), have shown that cold resistance in garden and sugar beet seedlings is increased by treatment with Dalapon (sodium 2,2-dichloropropionate) and TCA (sodium trichloroacetate). In earlier field tests with parsnips, increased resistance to fall frosts was observed after treatment with certain chemicals; namely, Colour Set (2,4,5-trichlorophenoxypropionic), and App-L-Set (sodium naphthaleneacetate) (56). However, Corns (8) found no effective increase in cold resistance in Dalapon-treated seedlings of Saunders wheat, Polish rape, Earliana tomatoes and Redwing flax. Miller and Corns (44) studied the differences between chemically-treated and untreated sugar beet seedlings. They concluded that the main factors contributing to the superior cold resistance of Dalapon and TCA-treated seedlings were: increased rate of water loss, decreased total moisture content, and increased sugar content. They reported no appreciable differences in either total or water-soluble nitrogen content as a result of the various treatments.

Numerous investigators have searched for a possible correlation between relative hardiness in winter wheat varieties and simultaneous changes in their carbohydrate and/or nitrogen fractions. In many instances, sugar concentrations have shown parallel increases with hardening and close correla-

tions have been obtained between sugar content and relative hardiness of varieties. However, there have been numerous exceptions in which the relationship was not constant, which led Levitt (32), in his review of the subject, to conclude that sugar concentration was only one factor in frost resistance. Nevertheless, it was generally accepted that an increase in hardiness was usually accompanied by an increase in sugar content and a simultaneous decrease in starch if any were present.

The nature of the changes occurring in the various nitrogen fractions during the hardening period are not well understood. Newton (46) found an increase in water-soluble and amino nitrogen with hardening of winter wheat varieties, which were originally selected on the basis of their wide range in degree of winter-hardiness. The hardiest variety had the largest water-soluble nitrogen content, but the relationship was not uniform throughout the series. Janssen (27) reported similar increases in the water-soluble nitrogen of winter wheats as the temperature decreased throughout the winter. On the other hand, Dexter (14) observed that plants of a hardy variety of winter wheat, which had a high nitrogen content, did not harden in the dark at 2° C., although there was a considerable increase in soluble organic nitrogen in the plants during the cold period. Another set of low-nitrogen, high-carbohydrate plants hardened well in the dark with a similar increase in soluble nitrogen. It appeared that the hardening process was largely dependent upon the organic nutrition of

the plant, when photosynthetic activity was lacking. Under conditions of low temperature and light, both sets of plants hardened efficiently. The reported increases in soluble nitrogen were less, however, in plants kept in the light as compared to those kept in the dark. Dexter concluded that an increase in soluble nitrogen was not an indication of increased hardiness, and that there appeared to be a series of reactions which proceed in a plant at low temperatures regardless of whether the plant hardens or not.

It seems relevant to the subject at this point to mention Nightingale's observations, as reported by Tysdal (65). Nightingale found that when plants were transferred from long-day to short-day conditions an enormous increase in soluble nitrogen occurred in the short-day plants. Thus we have a similar change to that mentioned by Dexter without the benefit of low temperature.

Janssen (27) also found that an increase in soluble nitrogen coagulable by heat accompanied lowering temperature to the freezing point, below which it showed a marked decrease. The percentage of water-soluble, coagulable nitrogen was greatest in the hardiest plants prior to freezing, after which time the coagulable nitrogen was considerably less than in the more tender plants. Janssen concluded that the hardier plants have a greater capacity for changing the protein nitrogen from a precipitable to a non-precipitable form. Newton, Brown and Anderson (49) observed a decrease in coagulable protein nitro-

gen after exposing press juice of unhardened winter wheat plants to frost. A concomitant increase in amino nitrogen led them to suggest that protein splitting occurred as a result of freezing.

More recently, Siminovitch and Briggs (57) observed a correlation between the changes in water-soluble protein nitrogen and hardiness of the live bark of black locust trees (Robinia spp.). Similar results have been reported since by Bula, Smith and Hodgson (5) working with alfalfa, and Hodgson and Bula (26) in their studies with sweet clover (Melilotus spp.). These results are in direct contrast with those reported by Janssen in wheat, although critical comparison in this case may not be valid.

With the exception of Konovalov's studies, as reported by Murneek and Whyte et al (45), there appear to be few data available with respect to nitrogen analyses of vernalized material. Konovalov determined total insoluble, soluble, amino, amide and ammoniacal nitrogen in seeds and plants during vernalization and under conditions preventing vernalization, and found considerable variation in their behaviour. With prevention of vernalization, the breakdown of the proteins extended to the end products, whereas during vernalization the proteins retained their form, but became more readily soluble. Konovalov concluded that nitrogenous substances appeared to be resynthesized during vernalization and considered this transformation to be a distinctive feature of the vernalization process.

According to the same authors (45), Pasevic observed that vernalization induced changes in the protein substances of the wheat germ, both the colloidal state and the amino-acid content of the proteins being affected.

In general, it appears that at low temperatures, and in some instances under short-day conditions, proteins are broken down into simpler nitrogenous compounds, regardless of whether the plant hardens or not. On the other hand, the possible causal relationship between water-soluble protein content and degree of hardiness appears promising, but remains unproven until the exact mechanism is known whereby the tissues avoid injury by extracellular freezing (59). Whether a similar relationship exists in winter wheats has not been demonstrated as yet.

Vernalization or low-temperature exposure of plants during germination in darkness may also be regarded as a form of hardening treatment. Dexter (12) has shown that marked hardening may occur in the dark in winter wheat plants exposed to constant low temperature, near 0° C., if there is abundant storage of organic food. Under the circumstances, the initial stages of vernalization might be expected to show an increase in total soluble nitrogen. With continuing low-temperature treatment (increasing hardiness), an increase in soluble protein might also be expected. However, the re-synthesis of nitrogenous substances during vernalization, as suggested by Konovalov, appears questionable.

EXPERIMENTAL

I. Vernalization

1. Materials and Methods

The varieties selected for study are listed below in order of decreasing winter-hardiness, as determined by winter survival data obtained at the University of Alberta, Edmonton, Alberta. Henceforth, varieties will be referred to as designated, and will be tabulated in order of decreasing hardiness unless otherwise stated. Classification (6) and other details are as follows:

Variety	Designation	Hardiness rank	Classification
Kharkov-M.C.22	MC	1	hard red winter - hardy
Minhardi*	M	2	soft to semi-hard red winter - "
Yogo	Y	3	hard red winter - "
Minturki	MT	4	semi-hard red winter - "
Kharkov-1442	K	5	hard red winter - "
Jones Fife	JF	6	soft to semi-hard red winter - semi-hardy
Vigo*	V	7	" " " - "
Seneca*	S	8	" " " - non-hardy
Thatcher	T	9	hard red spring - "

* Seed obtained from R. M. Caldwell, Purdue University, Lafayette, Indiana.

Observations on winter survival at Edmonton indicate that the varieties MC, M and Y are about equally winter-hardy. The varieties MT and K also exhibit small differences in hardiness, their percentage of winter recovery being approximately 10% less than MC, M and Y.

The vernalization technique employed was as follows: An equal number of seeds of each variety were weighed and disinfected with 0.3% H_2O_2 solution for 5 minutes. The seeds were then washed several times with sterile, demineralized water and placed on filter paper in Petri dishes. The Petri dishes, covers and filter paper were sterilized and weighed prior to use. After soaking the grain in a slight excess of water for 24 hours at room temperature, the surplus water was removed, seeds whose sprouts had not pierced the seed coat were discarded, and the remainder were transferred to a cold room, where they were maintained at 2° C. in darkness.

The treatments consisted of the varieties listed above, vernalized at 10-day intervals for periods ranging from 0 to 90 days, with the exception of JF, where the periods of treatment did not extend beyond 60 days. The various lots of seed were weighed twice weekly, and the moisture content of the grain maintained as close to 75% as possible. At no time did the moisture content fall below this level. Seeds were exposed to light only briefly during weighings, at which time diseased kernels were removed. After completion of vernalization, the treated material was transplanted in greenhouse beds.

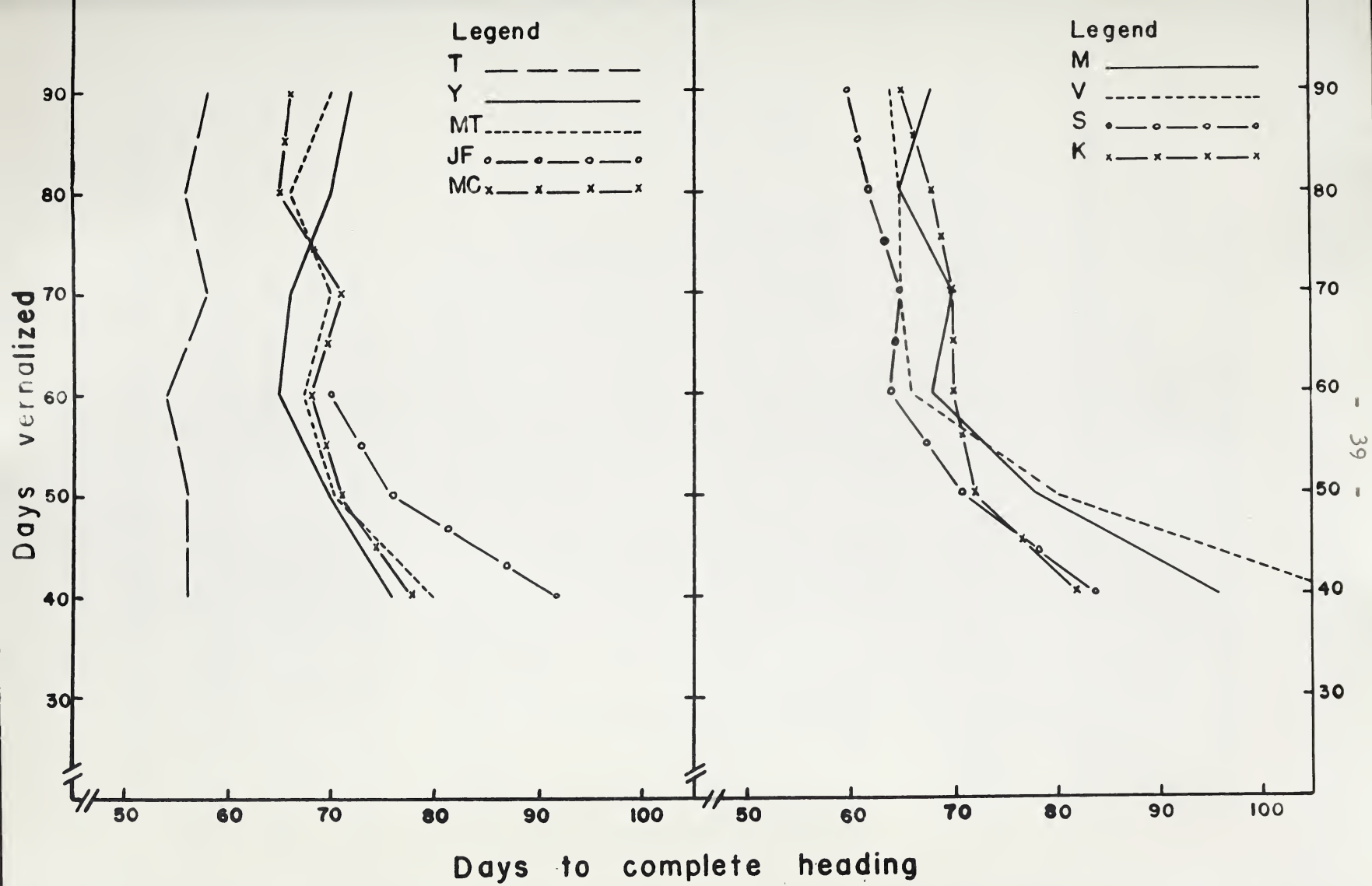
Each treatment was divided into 2 lots and the treatments were randomized in each of 2 replicates, the latter being planted on consecutive days.

The material was grown under natural day-lengths in the greenhouse, the planting dates being April 6 and 7, 1955. The temperature, which was set for 21.1° C. (70° F.), frequently rose to a high of 32.2° C. (90° F.) during the latter part of June, and July and August. The number of days from emergence to shooting, initiation of, and 50% heading, were recorded in all treatments. The number of days to complete heading was obtained in the 40 to 90-day-vernalized treatments, inclusive. The average numbers of leaves and tillers per plant were obtained from individual plant counts.

2. Results

Figure 1 shows the effects of various periods of vernalization upon the number of days required for complete heading of the varieties under the conditions of the test. It is noteworthy that beyond the 60-day-vernalization period there is little decrease in the number of days required for complete heading in all winter varieties. Six of the 8 varieties appear to be fully vernalized after 60 days. Furthermore, regardless of varietal hardiness, the maximum time required for complete vernalization appears to be 60 days.

Figure 1. The effect of various periods of vernalization upon days to complete heading of several wheat varieties.


Legend
T
Y
MT
JF
MC
Legend
M
V
S
K
Days vernalized
90
80
70
60
50
40
30
50
60
70
80
90
100
Days to complete heading

Additional observations on the same material are presented in table 1. Intervarietal responses to earliness, as indicated by the range in days to heading, appear to be less pronounced within the 60 to 70-day-vernalized treatments.

Table 1. The effect of various periods of vernalization upon rate of development in certain winter wheat varieties.

Vernalization treatment	Mean days to heading of all varieties	Total days to heading including previous low-temperature period	Range in days to heading of all varieties	Variations in range (days)	Mean dates of heading
90 days	66	156	60-72	12	June 12
80 "	66	146	62-70	8	" 12
70 "	69	139	65-71	6	" 15
60 "	67	127	64-70	6	" 13
50 "	74	124	70-80	10	" 20
40 "	87	127	76-108	32	July 3

In agronomic tests concerned with times of heading, it is often convenient to record the number of days to 50% rather than to complete heading. Such observations were made on the varieties referred to above and are illustrated in graphical form in figures 2 and 3. At this stage of maturity, the varietal response curves show the periods required for

Figure 2. The effect of various periods of vernalization upon days to 50 per cent heading of several wheat varieties.

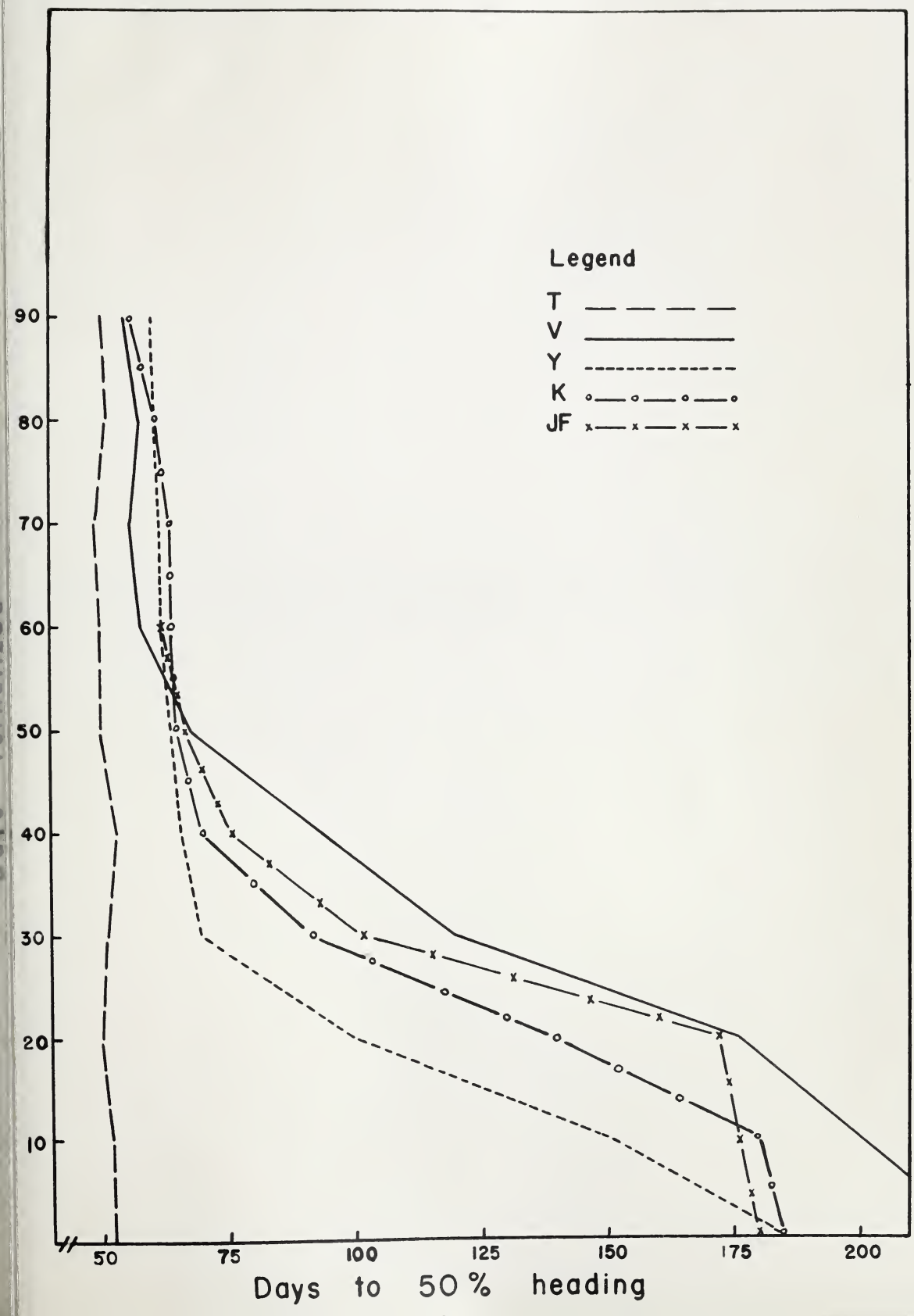
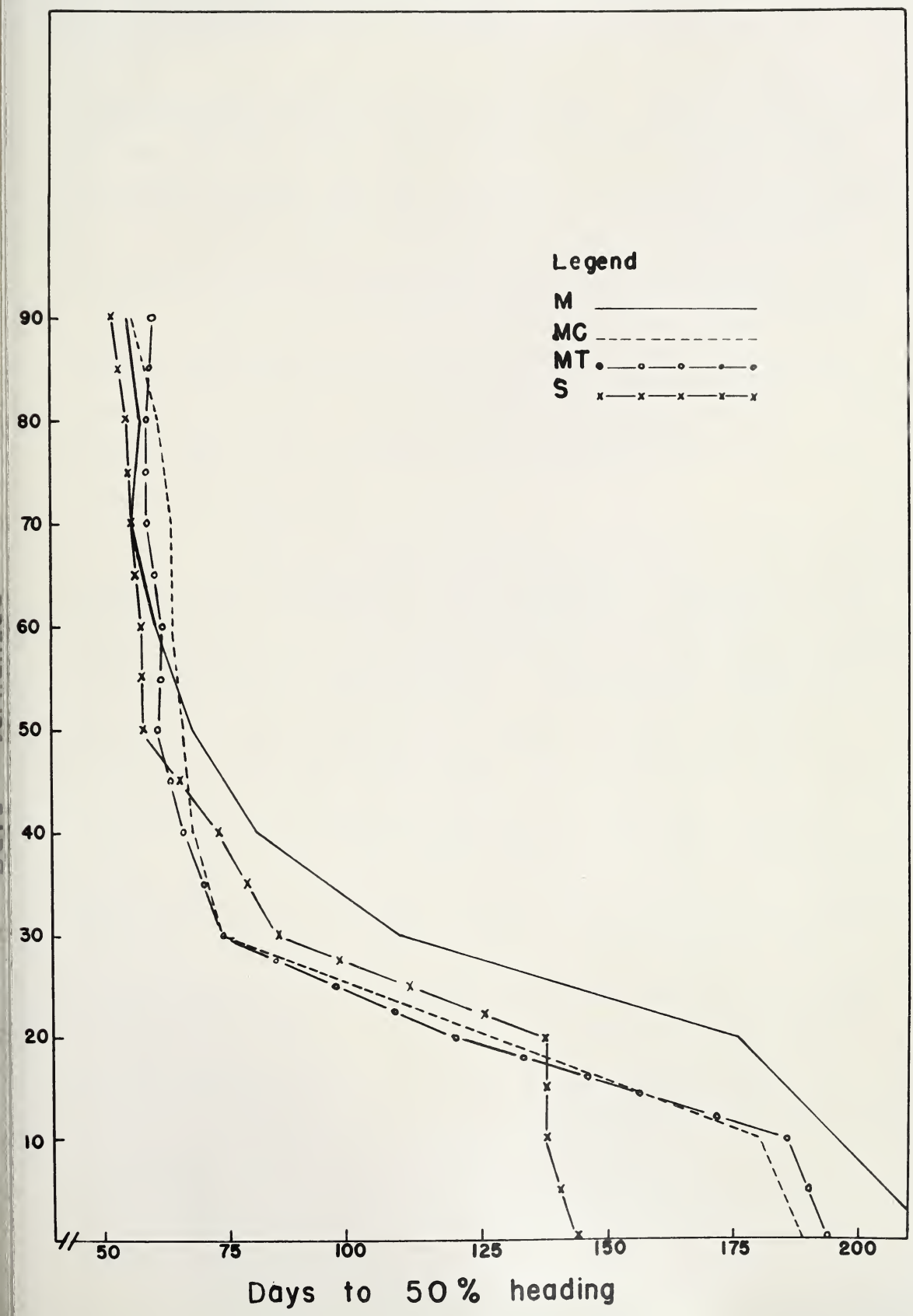

Legend
T
V
Y
K
JF
90
80
70
60
50
40
30
20
10
50
75
100
125
150
175
200
Days to 50 % heading

Figure 3. The effect of various periods of vernalization upon days to 50 per cent heading of several wheat varieties.


Legend
M
MC
MT
S
90
80
70
60
50
40
30
20
10
50
75
100
125
150
175
200
Days to 50% heading

vernalization to vary from 50 to 70 days. Despite these variations, however, there appears to be no relationship between varietal hardiness and time required to complete vernalization. One of the more tender varieties, V, appears to need 70 days to achieve the fully-vernalized condition, and the hardy variety, M, seems to need an equal period of time. Observations made at the fully-headed stage, however, suggest the optimum vernalization period for M to be 60 days, which indicates that counts made prior to completion of heading are apt to be misleading in such tests.

Leaf and tiller counts were made also on individual plants at the completion of the experiment. The average numbers of leaves and tillers per plant for the various treatments are summarized in tables 2 and 3. The number of leaves per plant on the main culms varied between minimum and maximum values of 5 and 22, respectively. A minimum leaf value of 5 in wheat has been reported previously by McKinney and Sando (41). The average leaf values obtained in this study remained generally constant for each variety after completion of vernalization. Although the differences in leaf counts for the spring variety were practically negligible, gradual but marked increases in number of leaves for the winter varieties accompanied reduced periods of vernalization. These effects are reflected in statistically significant differences obtained for treatments and for the interaction of treatments x varieties. Tillering, on the other hand, was not extensive in any treatment.

Table 2. Average number of leaves per plant at heading of varieties vernalized for various periods.

Variety	Number of days vernalized									
	90	80	70	60	50	40	30	20	10	0
MC	6.5	6.8	7.2	7.5	7.4	7.9	9.6	12.0	15.6	17.8
M	6.2	6.4	6.6	7.2	7.3	8.7	12.0	13.2	17.6	17.8
Y	6.2	6.8	7.0	7.1	7.2	7.8	9.1	9.4	14.6	16.0
MT	6.4	6.7	6.6	7.0	7.2	8.0	10.0	11.4	15.8	16.9
K	6.8	7.1	7.4	7.4	7.7	7.6	11.2	12.2	15.0	17.0
JF	-	-	-	8.2	8.4	9.8	11.2	14.7	17.2	17.8
V	6.8	6.5	7.0	7.6	7.8	10.4	12.4	14.6	15.4	17.8
S	6.4	6.4	6.6	7.4	8.4	9.6	10.7	14.0	13.8	15.2
T	6.0	5.2	5.4	5.8	6.0	6.4	6.9	6.8	6.9	6.8

Table 3. Average number of tillers per plant at heading of varieties vernalized for various periods.

Variety	Number of days vernalized									
	90	80	70	60	50	40	30	20	10	0
MC	1.02	1.02	1.05	1.12	1.14	1.05	1.36	1.22	1.54	1.68
M	1.16	1.12	1.10	1.00	1.40	1.24	1.16	1.02	1.41	1.41
Y	1.00	1.00	1.04	1.05	1.04	1.02	1.06	1.04	1.44	2.02
MT	1.02	1.00	1.04	1.00	1.29	1.00	1.30	1.18	1.34	1.59
K	1.14	1.24	1.02	1.04	1.02	1.12	1.63	1.18	1.34	1.85
JF	-	-	-	1.02	1.57	1.32	1.05	1.02	1.02	1.14
V	1.02	1.00	1.00	1.00	1.12	1.24	1.44	1.14	1.22	1.40
S	1.00	1.00	1.00	1.02	1.05	1.20	1.12	1.10	1.52	1.35
T	1.00	1.00	1.00	1.00	1.00	1.02	1.02	1.00	1.00	1.00

A more detailed study of the effects of various periods of vernalization within the hardy variety, MC, was also undertaken at this time. Seeds were vernalized at 2-day intervals, ranging from 0 to 86 days, as described previously. Following completion of vernalization treatment, the material was again divided into 2 lots, the treatments were randomized in each of 2 replicates and transplanted in the greenhouse on April 18, 1955. The plants were grown in one end of a greenhouse bed, the remaining portion of which contained material from the larger experiment described above. Data on the average number of tillers and leaves per plant, as well as the number of days to 50% and 100% heading, are given in table 4.

Complete vernalization appears to be achieved in the material given 50 days' cold treatment prior to planting. This vernalization period is 10 days shorter than that observed with the same variety in the previous test (see figure 1). Whereas the 11-day differential in planting dates has been maintained in the comparable 60-day treatments of MC through to complete heading, the 50-day treatment in the single variety test, by comparison, has shown a decrease in time to 100% heading, of 4 days. Direct comparison with its counterpart in the earlier test shows that the initial difference of 11 days in planting time has been practically annulled. Comparing tables 1 and 4, it is seen that plants of the 50-day treatment headed on consecutive days. It would appear that the combined effect

Table 4. Response of Kharkov M.C.22 to various periods of vernalization.

Number of days vernalized	Average number of tillers per plant	Average number of leaves per plant	No. of days to 50% heading	No. of days to 100% heading	Date of complete heading
80	1.00	6.3	59	65	June 22
70	1.02	6.6	57	64	June 21
60	1.00	7.0	57	67	June 24
50	1.00	6.8	57	64	June 21
40	1.07	7.5	63	68	June 25
30	1.38	8.8	76	92	July 19
20	2.04	9.0	105	120	Aug. 16
10	2.29	12.2	162	190	Oct. 25
0	2.83	14.6	211	215	Nov. 19

Planting date - April 18.

of increasing day-length and the slightly higher temperatures prevailing during growth (due to the effect of gradually increasing outdoor temperature upon greenhouse temperature) has caused a considerable increase in rate of development of plants of certain treatments.

3. Discussion

The apparent discrepancies in time to achieve complete vernalization, as observed in treated material of the

same variety when sown on different dates, may perhaps be explained by Friend's and Gregory's work with winter rye. It appears that once a winter rye plant becomes fully vernalized subsequent heat treatments are incapable of causing further devernalization. Friend and Gregory (16), however, found an acceleration in time to flowering with prolonged heat treatments (3 to 6 weeks at 20° and 25° C.) in partially vernalized winter rye plants. Since the 60-day-treated plants of MC are comparable in both tests, we might assume that they were fully vernalized prior to planting and thus would not be seriously affected in their flowering behaviour by subsequent high-temperature treatment. At the same time, prolonged high-temperature exposure might cause an acceleration in the rate of development of 50-day-vernalized plants if we assume their partial vernalization. Whether similar conditions exist in winter wheat as have been observed in winter rye is not known. The situation is highly suggestive, however, of such a possibility. If such were the case, it is evident that all vernalization treatments below a certain threshold value would be affected in their flowering behaviour under similar circumstances. Without rigid experimental controls, however, the extent and manner of such effects are highly speculative. The controls employed in these particular tests do not permit detailed conclusions. The gradual increase and subsequent decrease in length of photoperiod up to and beyond June 21, and the wide variations in temperature existing throughout the experiments, serve to illustrate this fact.

The possibility of vernalization before seed ripening in the field must also be considered with regard to the seed-stocks used. Kostjucenko and Zarubailo are reported by Whyte (68) to have noted such effects in developing embryos of winter wheat plants prior to final ripening. Similar effects, reported by Gregory and Purvis in winter rye plants, have been mentioned previously. It has also been noted that hardiness in winter wheats appears to decrease with increasing periods of vernalization. If this is so, and if, as Whyte suggests, the inherent cold resistance of individual varieties varies from year to year, depending upon the degree of pre-dormancy development, the task of evaluating the relative hardiness of varieties maturing under generally cold conditions might be exceedingly difficult.

On the other hand, seeds which were ripened under conditions of warm days and cool nights might be expected to show little vernalization effect, if the occurrence of such an effect during the cooler periods were overbalanced by the de-vernalizing effect of the warmer-day temperatures. Such a situation might apply to the winter varieties under test; namely, MC, Y, MT, K and JF, which were ripened at Edmonton in late July and early August of 1952. As mentioned previously, the remaining winter varieties, M, V and S, were obtained from Northern Indiana, where little if any pre-dormancy vernalization might be expected. The temperature conditions obtaining at Edmonton from July 15 to August 15, 1952, show a mean high of

72.5° F., and a mean low temperature of 52.0° F. During this period, the temperature decreased to 44° F. on two occasions and dropped below 50° F. (10° C.) 9 times in all. For the same period, a maximum temperature of over 80° F. was recorded 6 times. Under the temperature conditions which prevailed during the ripening period, one would expect a negligible vernalization effect in the varieties concerned. While prolonged periods of cool, damp weather during ripening might exert a considerable vernalizing effect at this time, it should be noted that the pronounced pre-dormancy vernalization effects observed by Kostjucenko and Zarubailo occurred at a latitude of N. 67° 44', which is more than 800 miles north of Edmonton.

The data in figures 1 to 3 show that frequent reversals occur in the relative order of heading of certain varieties. If the graph from one section of figure 1 were superimposed on the adjacent portion, this phenomenon would be still more evident.* Similar observations are reported by McKinney and Sando (41), and Gries et al (21). The former authors were of the opinion that differences in the environment-response characteristics of the growth phases of the varieties were largely responsible for reversals in the order of heading. They believed that these characteristics might be measured indirectly in terms of internode number, growth rate, and in time

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* The differential response of varieties x treatments was statistically highly significant.

of flowering over a range of temperature and light conditions.

Gries et al (21) studied the response of 4 spring wheat varieties under controlled conditions of temperature and photoperiod. They found that the influence of temperature on the response to photoperiod varied according to varietal characteristics. While increasing photoperiods promoted increasing earliness in all varieties, maximum earliness was obtained under a particular optimum temperature, depending upon the variety. Fully-vernalized winter varieties, however, while requiring long days for early attainment of flowering, may respond better under relatively lower optimum temperatures than spring varieties.

The irregular pattern of the response curves illustrated in figures 1, 2 and 3 indicate the existence of wide intervarietal differences in behaviour with respect to earliness, even in fully-vernalized material. It is impossible, however, under the variable conditions of these studies, to assess with any certainty the individual effects of such variables as temperature, day-length and vernalization treatment.

II. Freezing Tests

1. Materials and Methods

Seedlings tested in the one-leaf or coleoptile stage of growth were grown in (a) sterilized Petri dishes containing moistened filter paper, and (b) Petri dishes containing a measured amount of vermiculite and distilled water. To reduce the incidence of disease and of mould contamination, seeds were placed in Gooche crucibles, prior to planting, and soaked in Orthocide solution (0.5 gm. per l.) for 5 minutes. This was followed by drainage of excess solution, and planting, as described above, in a germinating cabinet maintained at 22.2° C. (72° F.) and high humidity. Seed treatment prior to planting, in some of the earlier experiments, consisted of soaking for 5 minutes in 0.3% H_2O_2 solution. This treatment was eventually replaced in favour of the additional protection afforded by Orthocide (the active ingredient of this seed protectant is N-trichloromethylthiotetrahydrophthalimide).

Seedlings tested in the 2 to 4-leaf stage of growth were grown in clay pots, $6\frac{1}{2}$" in diameter, containing a measured amount of sterilized soil and sand mixture in the proportion of 3:1, respectively, in a greenhouse maintained at approximately 21.1° C. (70° F.). After treatment in Orthocide solution, the seeds were planted in a circle, equi-distant from the edge of the pot and from each other, at a depth of $1\frac{1}{2}$ inches. Each planting consisted of several seeds, the seedlings being thinned

after emergence to 12 plants per pot.

When seeds were germinated in Petri dishes containing filter papers, the latter were kept as uniformly moist as possible throughout both high and low-temperature treatment. The maintenance of moisture level during vernalization has been referred to previously. It should be borne in mind that demineralized, rather than distilled, water was used in all vernalization tests. Slight moisture excesses were drawn off by vacuum prior to freezing in all such experiments. Where vermiculite was employed as a growth medium, the initial watering sufficed through germination and freezing. Distilled water was then added after thawing at room temperature to aid recovery of the seedlings. Unless otherwise stated, this procedure was adhered to in treatments utilizing vermiculite. Tap water was added in excess at regular intervals to greenhouse seedlings grown in pots. Following removal to the hardening chamber, a measured amount of distilled water was added periodically. In critical tests, care was taken to equalize the water content of the various treatments by the addition of excess water approximately 16 hours prior to freezing.

Samples which were subjected to rapid freezing were placed on a slowly revolving metal tray, which was mounted in one end of a large deep-freeze unit and driven by a small electric motor. The speed of revolution of the tray was controlled by a rheostat. Minimum air temperatures were read from a stationary thermometer placed directly adjacent to, and

level with the tray which contained the material to be frozen.

The majority of the tests involving hardening and slow freezing were carried out in an illuminated, controlled-temperature room (7'4" x 12'6" x 7'6"). A small cabinet unit was used for a few of the earlier tests before the larger room became available. Details of individual experiments will be dealt with in order.

2. Results

(1) Unhardened material

The following tests have been selected as being more or less representative of numerous other freezing tests with unhardened material. Seeds of 8 varieties of wheat were distributed evenly in heavy flat-bottomed Petri dishes containing a measured amount of vermiculite. There were 45 seeds per dish, and 4 replicates per treatment. After distributing the seeds, a further measure of vermiculite was added to each dish, before tamping down firmly and adding sufficient water to saturate the material. The Petri dishes were then placed on metal trays in a germinating cabinet maintained at 22.2° C. in darkness. Four days after emergence, at a height of approximately 50 mm., 25 seedlings from each replicate of each variety, selected for uniformity, were severed immediately above the seed-piece and attached basally to strips of Scotch

tape stretched across small wire frames according to the method developed by Corns (8). Four frames, each containing 50 detached coleoptile sections were placed on the revolving metal tray and frozen simultaneously in the deep-freeze unit. After the desired freezing period had elapsed, the samples were removed to a side bench and the percentage recovery was determined from the number of shoot sections which did not collapse with thawing at room temperature. Table 5 illustrates the effects of repeated exposure to a temperature of -15° C. for 8 and 12

Table 5. Percentage recovery of unhardened coleoptile sections after subjection to a temperature of -15° C. for 8 and 12 minutes.

Variety	Hardiness ranking from winter-survival data	Percentage recovery after exposure to -15° C. for:		Hardiness ranking after exposure to -15° C. for:	
		8 min.	12 min.	8 min.	12 min.
MC*	1	17	0	8	8
M	2	75	62	3	2
Y	3	43	23	6	6
MT	4	48	37	5	5
K	5	52	46	4	4
V	6	80	69	1	1
S	7	78	60	2	3
T	8	22	19	7	7

* Mould contamination.

minutes, respectively.

It is evident that the hardiness ratings, indicated by the survival data from unhardened material, bear little resemblance to the actual order of hardiness as determined from winter survival tests. Two of the more winter-tender varieties, V and S, show the least injury after 8 minutes' cold exposure; and except for the reversed position of S and M after 12 minutes cold treatment, the former varieties still rank first and third, in order of maximum survival. However, with the removal of MC from the test, as noted, we do find the spring variety, T, in its correct position as the least cold-resistant.

In a further test with essentially unhardened material, the 8 varieties were grown in flats in the greenhouse, 8 rows per flat and 50 seeds per row, replicated 8 times. The seeds were planted at a depth of 1½ inches in a measured amount of sterilized soil and sand, mixed in the proportion of 3:1, respectively. After emergence, the varieties were thinned to 30 plants per row, and it was decided to freeze 2 flats at intervals of 1, 2, 3 and 4 weeks after emergence. A final thorough watering was applied 24 hours prior to freezing, at which time the flats were removed to a cooling chamber maintained at approximately +5° C. Freezing was accomplished in the deep-freezer at the slowest rate attainable. When the desired temperature was reached, the freezing unit was dis-

connected and the temperature was permitted to rise slowly. This procedure was adopted in all but the first treatment (earliest growth stage), in which case the lid was left slightly ajar. The percentage recovery, based on the number of plants surviving 2 weeks after freezing, is given in table 6.

Table 6. Percentage recovery of seedlings exposed to varying periods of freezing treatment 1, 3 and 4 weeks after emergence. (Hardiness ranking in brackets.)

Interval emerged	1 week	3 weeks	4 weeks
Stage of growth	2-leaf	3 to 4-leaf	3 to 4-leaf
Duration of exposure	5¼ hr.	13 hr.	13 hr.
Minimum air temperature	-9.5° C.	-8.0° C.	-8.0° C.
Variety - MC	83 (2)	27 (4)	76 (3)
M	32 (8)	28 (3)	75 (4)
Y	85 (1)	3 (8)	60 (5)
MT	72 (4)	34 (2)	83 (1)
K	77 (3)	50 (1)	82 (2)
S	51 (7)	23 (5)	36 (6)
V	53 (6)	19 (6)	76 (3)
T	60 (5)	8 (7)	21 (7)

The material with a 2-week period of emergence (2 to 3-leaf stage), to which reference does not appear in the table, was subjected to a minimum air temperature of -12.5 C., the duration of exposure being 19 hours. Recovery in all cases was nil.

The results, as indicated above, are erratic to say the least. With few exceptions, none of the varieties is found in the relative order of hardiness obtained from winter-survival observations. In general, most of the hardy varieties are included in the first five rankings, but there are exceptions in each treatment with inconsistent behaviour being exhibited throughout each variety. It is realized that soil-moisture content exerts an important influence on the rate of temperature decrease during freezing, and thus directly affects subsequent seedling survival. For this reason, care was taken to avoid immoderate drying following the final watering when the flats were well saturated with water and placed in the cooling chamber. Numerous investigators have pointed out the difficulty of avoiding small variations in moisture content, especially when flats are used in freezing tests. For this and other reasons, the use of flats was largely discontinued in further tests of this kind.

It was concluded that freezing tests with unhardened material were of little use in the differentiation of hardiness between varieties, particularly where only small intervarietal differences might exist.

(2) Vernalized material

In an effort to determine the effect of prolonged vernalization treatment on varietal hardiness, seeds of several varieties were vernalized for various periods of time and subjected to quick-freezing. Prior to freezing, a uniform number of seedlings were retained in individual Petri dishes and the excess moisture was withdrawn from the filter papers by vacuum. Following removal from the deep-freezer, the Petri dishes were placed on side benches to allow the material to thaw at room temperature. After approximately 30 minutes, the treatments were watered and covered to prevent excessive evaporation. Survival counts were made on the seedlings 6 to 7 days after freezing. The intact seedlings appeared quite vulnerable to freezing, and in many cases injury appeared to be progressive. In spite of this, it was felt that recovery counts of apparently undamaged seedlings should be fairly indicative of hardiness, even though death might eventually occur. The data showing percentage recovery, after 12 minutes' exposure to a temperature of -20° C., are summarized in table 7.

In the initial stages and up to 32 days, the increase in cold resistance appears slight in the winter varieties and lacking in the spring variety. Following this period, there is a rapid increase in hardiness, especially in the more tender winter variety, S. Beyond the 58-day period and coincident with completion of vernalization, a considerable decrease in cold resistance occurs, this being more pronounced

Table 7. Percentage recovery of vernalized seedlings after exposure to -20° C. for 12 minutes.

Vernalization period	Varieties				
	MC			S	T
10 days	0			0	0
22 "	0			0	0
32 "	16			14	0
52 "	53			90	38
58 "	100			100	100
	M	K	JF		
70 days	60	60	30	30	0
80 "	50	50	30	10	0

in the more tender varieties, and less so in the hardier types. The marked increase in resistance to cold in the spring variety, T, prior to the 58-day period, and the equally striking decrease which follows, is rather surprising. Since vernalization is unnecessary for the rapid induction of flowering in this variety, the eventual loss of hardiness generally occurring in the winter varieties after vernalization does not serve to explain too satisfactorily the exceedingly rapid loss of hardiness in T.

(3) Short-term hardening (up to 15 days)

On numerous occasions, seedlings of several wheat varieties were germinated in vermiculite and kept for short periods at 22° C., and subsequently hardened for intervals of from 48 to 96 hours at a constant temperature of 0° C. under continuous but weak illumination. Recovery counts of severed shoots, after attachment to tapes stretched over wire frames, and subjection to a temperature of -15° C. for periods up to 12 minutes in the deep-freezer, were erratic and showed little or no relation to actual winter survival rating.

In other tests, equal numbers of seedlings, grown under similar conditions and hardened for periods up to 72 hours, were selected for uniformity and placed in Petri dishes with moistened filter paper prior to freezing for periods up to 12 minutes at -20° C. In general, the winter varieties classified as hardy showed less damage than the more tender winter types, but the relative hardiness ratings within both types were inconsistent. However, the tender spring variety frequently exhibited as little or less injury than the tender winter types, and in some cases as little injury as the hardier varieties. When intact seedlings were subjected to temperatures as low as -20° C. for short periods, it was difficult to obtain the preferred degree of injury due to the severe freezing conditions. With few exceptions, damage was either negligible or severe enough to cause ultimate death. While notes on apparent re-

covery, taken several days after freezing, served to act as a guide to indicate intervarietal differences in cold resistance, actual recovery remained unproven in most cases. For this reason, the results are open to criticism.

Further tests included freezing of seedlings in Petri dishes containing vermiculite. After 5 days' germination in this medium at 22.2° C., uniform numbers of seedlings were maintained for periods up to 14 days at a constant low temperature in both darkness and continuous light (150 W. approximately 4 feet distant), prior to freezing. After removal from the deep-freezer and thawing for 30 minutes at room temperature, nutrient solution was added to all samples. The results of one such test, which were fairly representative, are shown in table 8. Recovery counts were made 10 days after freezing. The average height of the seedlings is shown in brackets.

Intervarietal differences in degree of recovery are evident in the material which has been exposed to -20° C. for 18 minutes (column 2), where the varieties are seen to be in the accepted order of hardiness. Duplication of results by this method is difficult, however, due chiefly to inequalities in moisture between samples. The wheat hardened for 14 days shows little, if any, increase in hardiness over that of seedlings hardened for 7 days. Similar results have been reported by Suneson and Peltier (63). They attributed this effect to

Table 8. Relative cold resistance of germinating wheat seedlings after constant low-temperature treatment (2° to 3° C.) in darkness for varying periods of time.

Exposure time at -20° C.	15 min.	18 min.	20 min.
Hardening period	7 days	7 days	14 days
Variety	Percentage recovery		
M	100 (70 mm.)	85 (70 mm.)	25 (80 mm.)
Y	100 (80 mm.)	75 (80 mm.)	0 (90 mm.)
JF	100 (70 mm.)	70 (70 mm.)	0 (85 mm.)
T	0 (70 mm.)	30 (70 mm.)	0 (85 mm.)

endosperm exhaustion. Dexter (12) found that winter wheats hardened quite well in darkness at constant low temperature (0° C.), provided a plentiful supply of organic food was present. The results obtained here support this view.

After 5 days' germination in vermiculite at room temperature, seedlings of the same varieties were hardened in continuous light for various periods and exposed to three methods of freezing. In method a, uniform numbers of seedlings were frozen in Petri dishes with vermiculite; in method b, intact seedlings were exposed in Petri dishes; and in method c, severed coleoptile sections were frozen by means of the tape technique. Recovery was based on the percentage of leaf-tip

killing 5 days after freezing in method a, the numbers of dead seedlings in method b, and the number of shoot sections collapsing after thawing in method c. The results are given in table 9.

Table 9. Percentage recovery of germinating wheat seedlings exposed to various methods of freezing subsequent to 7 and 14 days' hardening at 0° C. in continuous light. (The percentage recovery of unhardened control seedlings is given in brackets.)

Method	a	b	c
Hardened	7 days	7 days	7 days
Exposure time at -20° C.	15 min.	12 min.	10 min.
Variety	% recovery		
M	80 (20)	10 (0)	47 (13)
Y	90 (10)	0 (0)	27 (7)
JF	75 (15)	0 (0)	53 (13)
T	55 (10)	0 (0)	27 (7)
Hardened	14 days	14 days	14 days
Exposure time at -20° C.	25 min.	12 min.	10 min.
Variety	% recovery		
M	75 (5)	40 (30)	100 (0)
Y	45 (10)	40 (10)	100 (0)
JF	25 (0)	30 (20)	100 (0)
T	0 (0)	0 (0)	100 (0)

The "7-day-hardened" treatments in method a received an additional exposure to -20° C. of 15 minutes the day prior to final freezing; the 14-day treatments received an additional exposure to -20° C. of 18 minutes the day before the final freezing test.

Regardless of method of freezing, the "14-day-hardened" plants show a considerable increase in cold resistance over the "7-day-treated" plants, with the exception of the spring variety, T, in which the hardening response appears to be limited. Intervarietal differences in degree of recovery corresponding to actual winter survival ratings are evident in the varieties hardened for 14 days and frozen in Petri dishes with vermiculite (method a). Varietal differences in hardiness are also evident with the shorter period of hardening, in which case Y appears hardier than M by method a, but less hardy than M by methods b and c. While this discrepancy might be attributed to relative differences in freezing injury due to variations in moisture between samples in method a, the reversed hardiness ranking of M and Y (apparent by this method with an additional 7 days' hardening) suggests that varietal differences in rate of acquisition of hardening might also account for the observed change.

In method b, differential injury between varieties is only apparent after 14 days' hardening, which suggests that the freezing treatment employed with "7-day-hardened" material

was too severe to show possible differences in cold resistance. For the same reason, increased exposure to freezing appears necessary in method c with "14-day-hardened" material to indicate intervarietal differences in hardiness. Further testing also appears necessary to explain the inconsistent hardiness ratings obtained in "7-day-hardened" plants with all three methods of freezing.

In a preliminary experiment with potted material, wheat plants of 4 varieties were grown in the greenhouse and subjected to alternating high and low temperatures for periods of 5, 10 and 15 days prior to freezing. The hardening treatment consisted of 8 hours' growth at approximately 24° C. (75° F.) under natural daylight and 16 hours at 4.5° C. (40° F.) in weak illumination (100 W. at a distance of 8 feet). Pots were watered in excess at regular intervals up to the day prior to freezing. The various hardening treatments were completed simultaneously, at which time all plants were in the 3 to 4-leaf stage of growth. Individual treatments of the 4 varieties were then exposed in the deep-freezer for periods of 20, 40 and 60 minutes on consecutive days. Following each exposure, the plants were thawed, watered and left on side benches at room temperature. After completion of the final freezing, the plants were removed to the greenhouse, where freezing injury, based on the total number of leaves, was assessed after 7 days. The results, expressed as percentage recovery, are shown in table 10.

Table 10. Percentage recovery from freezing of wheat seedlings after various periods of hardening at alternating temperatures. (Hardiness ranking shown in brackets.)

Treatment	Control unhardened	5 days hardened	10 days hardened	15 days hardened
Average freezing air temperature	-14.0° C.	-15.0° C.	-15.5° C.	-16.0° C.
Variety	% recovery			
MC	61 (1)	44 (3)	65 (1)	58 (3)
MT	38 (3)	62 (1)	25 (4)	67 (1)
S	55 (2)	39 (4)	54 (2)	64 (2)
T	34 (4)	54 (2)	50 (3)	40 (4)

The average heights of the seedlings prior to initial freezing are tabulated below in centimetres.

Treatment	Control unhardened	5 days hardened	10 days hardened	15 days hardened
Variety				
MC	27	22	22	16
MT	28	24	24	16
S	28	26	18	18
T	26	26	22	18

Fluctuations in freezing temperature at various positions within the deep-freezer were a definite source of error in this experiment, and for this reason comparisons within treatments are not strictly valid. Temperature variations within the freezer may account for the fact that varietal hardiness ranking does not correspond in any treatment to that found in winter-survival data. Although the varieties, MC and T, are found in their accepted order of hardiness, in this respect, on two occasions, the remaining two varieties do not conform to winter-survival ranking in any treatment. This might be attributed to lack of hardening, since varietal differences in cold resistance appear to become more pronounced with increased acquisition of the hardened condition. However, there appears to be a limited increase in cold resistance in the 15-day-hardened plants compared to the unhardened controls, although comparison between treatments is difficult due to the inability to maintain uniform temperature throughout prolonged freezing periods.

(4) Long-term hardening (3 to 6 weeks)

Subsequent to 48 hours' germination in Petri dishes containing moistened filter paper, at room temperature, seedlings of 4 varieties of wheat were subjected to a constant temperature of 0° C., in both continuous darkness and light (150 W. at an average distance of 4 feet), for periods of 3 and 4 weeks. When necessary, sufficient water was added to

maintain the filter papers as uniformly moist as possible. After completion of the hardening period, uniform numbers of each treatment of each variety, in each of 4 replicates, were transferred to Petri dishes which contained moistened filter paper. The dishes were maintained in the hardening chamber for approximately 30 minutes prior to exposure in the deep-freezer for 12 minutes at -20° C. Recovery counts taken 6 days after freezing are shown in table 11. The average height of the seedlings at freezing is also given.

Table 11. Amount of recovery, after 12 minutes' exposure at -20° C., of germinating seedlings grown for periods of 3 and 4 weeks at 0° C. in continuous light and darkness.

Variety	Percentage recovery			
	3 weeks' hardening		4 weeks' hardening	
	darkness	light	darkness	light
M	80	25	90	30
K	70	25	55	25
S	20	5	25	20
T	40	25	60	5
Variety	Average height of seedlings (cm.)			
M	14-15	5-6	16-18	6-7
K	10-12	3-4	14-16	5-6
S	8-9	3-4	8-10	5-6
T	16-18	5-6	18-20	6-8

Considerable differences, both in apparent recovery and in average height of seedlings at freezing, are evident between treatments hardened in darkness and in light. Large differences in percentage moisture between treatments hardened in light and in darkness, assumed on the basis of size disparity, would have an important bearing on the amount of freezing injury. With respect to size, the variety S, which is the least hardy in both 3 and 4-week treatments in the dark, also shows the least increase in height. Evidently the seedlings grown in darkness have either escaped freezing injury or they have acquired more cold resistance than seedlings grown in continuous light. In the latter treatments, the rate of increase in hardiness may have been limited by insufficient light since only a limited degree of hardiness seems to have been acquired.

The varieties are ranked in known order of winter survival in only one treatment, which is 4 weeks' growth at 0° C. in continuous light. Within both darkened and illuminated material, cold resistance appears to increase slightly with increased periods of hardening, but there are exceptions in both groups.

Five varieties of wheat were also grown to the 2 to 3 -leaf stage (20 days after planting) in pots in the greenhouse prior to hardening, for periods of 3 and 6 weeks at 0° to 1° C. with continuous light (300 W. at an average distance of 4 feet). The plants of each of 4 replicates were thinned, as

described previously, to a uniform number after emergence. During greenhouse growth, the plants were watered in excess daily. After removal to the hardening chamber, a measured amount of distilled water in slight excess was added to each pot every 5 days. Sixteen hours prior to freezing, an additional watering was supplied to equalize the soil-moisture content in each pot. Planting dates were staggered to permit simultaneous completion of hardening and freezing of both the "3 and 6-week-hardened" plants. The generally higher greenhouse temperatures which prevailed during the growth of the material which was later hardened for 3 weeks is reflected in the marked differences in average height between the two treatments, as shown in table 12.

It appears that the hardier varieties, MC and M, have a slower rate of growth, especially in the early stages of hardening, the differences becoming much less pronounced with increased exposure to low temperature. The comparatively high growth rate of the tender variety, T, suggests that relatively less energy is expended in the storage of reserve food compared to the winter varieties.

Prior to freezing, thermometers were inserted, to a depth of 1½ inches, in the soil in the centre of several pots. The controlled temperature dial of the freezing chamber was then set to -9° C.*, where it remained for 24 hours. Nine

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* The freezing temperatures recorded within the chamber were 1½ to 2 degrees lower than this setting.

Table 12. Average heights of 5 varieties of wheat plants in the 2 to 3-leaf stage at initiation and at completion of hardening for periods of 3 and 6 weeks at a temperature of 0° to 1° C. in continuous light. (Height measurement in millimeters taken from the second leaf.)

Variety	Treatment							
	3 weeks' hardening				6 weeks' hardening			
	Average height at initiation of		Average increase in height	Average growth rate mm./day	Average height at initiation of		Average increase in height	Average growth rate mm./day
	hardening	freezing			hardening	freezing		
MC	153	156	3	0.14	123	139	16	0.38
M	152	159	7	0.33	128	148	20	0.48
JF	162	174	12	0.57	144	166	22	0.52
S	147	158	11	0.52	126	147	21	0.50
T	193	206	13	0.62	151	189	28	0.67

hours after commencement of freezing, the minimum air temperature at the level of the pots, and the soil temperature at the stated depth in the pots, were -10.5° C. and -6.0° C., respectively. Twenty-four hours later, the minimum air and soil temperatures both read -10.5° C. At this time, the freezing unit was switched off and the pots were allowed to thaw gradually with the door of the controlled-temperature room slightly ajar. Seven hours later, the soil temperature of the pots had risen to 0° C. The pots were removed to the greenhouse the following morning (16 hours later), where a record of the amount of leaf damage was eventually taken. Seven days after freezing, individual leaves were classified as dead, partially injured (approximately 50% damage), tip-killed or uninjured. Twenty-one days after freezing, the total number of leaves, the number of severely injured leaves, and the number of dead plants, were recorded. The results are reported in tables 13 and 14.

Differences in degree of freezing injury were clearly observable throughout the "3-week-hardened" plants, especially between the hardy varieties (MC and M), the moderately hardy ones (JF and S), and the tender spring variety T. However, it will be observed that the relative hardiness rating of the "3-week-hardened" plants of JF and S is reversed in the 7-day and 21-day recovery counts. The reason for this change was the relatively greater production of new leaves in JF than in S following freezing, this being evident when leaf counts were

Table 13. Percentage recovery from leaf damage 7 days after freezing of wheat plants in the 2 to 3-leaf stage of growth, hardened for periods of 3 and 6 weeks in continuous light at a temperature of 0° to 1° C.

Variety	3 weeks' hardening		6 weeks' hardening	
	Recovery based on the number of leaves:		Recovery based on the number of leaves:	
	dead	dead + partially killed	dead	dead + partially killed
MC	100	98	100	100
M	81	72	100	100
JF	31	8	74	63
S	39	30	93	84
T	0	0	0	0

Table 14. Percentage recovery 21 days after freezing of wheat plants in the 2 to 3-leaf stage of growth, hardened for periods of 3 and 6 weeks in continuous light at a temperature of 0° to 1° C.

Variety	3 weeks' hardening		6 weeks' hardening	
	Recovery based on number of:		Recovery based on number of:	
	dead plants	severely injured leaves	dead plants	severely injured leaves
MC	100	81	100	90
M	92	71	100	91
JF	75	51	100	74
S	67	48	100	83
T	0	0	0	0

made 21 days after freezing. Where percentage recovery after 21 days was based on the proportion of severely injured leaves to total number of leaves, the reversal in hardiness rating between JF and S became evident. Comparatively greater stimulation of new growth following limited freezing may also account for the difference in survival after 21 days between JF and S, where recovery is based on the number of dead plants.

In the "6-week-hardened" material, differences in degree of hardiness between the hardy, moderately hardy and tender varieties are also distinct in all recovery counts but those based on the number of dead plants. It is apparent that the winter varieties have acquired a high degree of cold resistance after 6 weeks' hardening, and hence a more severe freezing test is needed to distinguish differences in hardiness on the basis of plants killed by freezing.

The data in tables 13 and 14 suggest that the hardy varieties, MC and M, acquire the hardened condition more rapidly than the other (less hardy) varieties, the effect being more pronounced in MC than in M. Worzella and Cutler (71) have reported similar results. The same authors suggest that soil temperatures of 0° to +5° F. (-17.7° C. to -15.0° C.) may be lethal to well-hardened wheat seedlings. Since the varieties above have been exposed to a minimum soil temperature of -11.0° C., with only limited killing on an individual plant basis, it appears that they have developed considerable cold resistance even after 3 weeks' exposure to the hardening treatment.

While recovery from freezing on the basis of plant survival is easy to determine, the desired degree of injury is not always obtained in this respect, as evidenced in the recovery of the "6-week-hardened" winter varieties based on the amount of plant killing (table 14). Since the assessment of individual leaf damage from freezing, as described above, is relatively easy to determine, providing the plants are not advanced beyond the 2 to 3-leaf stage at initiation of hardening, percentage recovery can usually be obtained on this basis; in which case the relative amount of injury is assessed more critically.

A modification of Newton's pressure method was also employed to determine the relative hardiness of the varieties. Newton (47) found that the quantity of juice expressed by pressure from the leaves of winter-hardened wheat plants was inversely related to their known winter-hardiness.

At the completion of hardening, weighed amounts of leaf material of each variety were placed between several layers of previously weighed filter paper and subjected to a pressure of 20,000 lb. per sq. in. Immediately following this treatment, the pressed material was removed and the filter papers were weighed as rapidly as possible. The difference in weight of the filter paper before and after pressure treatment permitted a calculation of the grams of moisture per gram of leaf material. The results are shown in table 15.

Table 15. Grams of moisture per gram of wheat-leaf material after subjection to a pressure of 20,000 lb. per sq. in. of varieties hardened for various periods at constant low temperature and continuous light.

Variety	Hardening period	
	3 weeks	6 weeks
MC	0.45	--
M	0.44	0.52
JF	0.50	0.52
S	0.50	0.56
T	0.57	0.48

With one exception, the amount of juice obtained from the leaves corresponds inversely to the known winter-hardiness rating of the varieties, the relationship being less evident in the "6-week-hardened" plants. Although the amount of material used in this test was much less than that used by Newton, the pressures employed were almost 4 times as great. A source of error in this preliminary test was the fact that the amount of material used in each test was not uniform, varying between approximately 1.5 and 2.5 grams. This discrepancy could affect the results considerably.

(5) Chemical treatments

In preliminary experiments, wheat seedlings were germinated in various concentrations of growth substances - notably, Colour Set (2,4,5-trichlorophenoxypropionic acid) and App-L-Set (sodium naphthaleneacetate) at concentrations of 10 to 20 p.p.m., and maleic hydrazide at much higher concentrations - for periods up to 9 days prior to freezing. Apparent survival, noted 4 days after freezing, suggested no increase in cold resistance over the control plants. However, in most cases the freezing tests were too severe to permit accurate appraisal of the results, so that further testing is necessary before definite conclusions can be drawn.

On numerous occasions, seeds of several wheat varieties were germinated in various concentrations of Dalapon (sodium 2,2-dichloropropionate) for several days and subsequently exposed for short periods in the deep-freezer to test for possible effects on cold resistance. In general, the results were erratic and inconclusive. The following experiments, which are more or less representative, serve to illustrate this point.

Seeds of the variety, MC, were germinated in Petri dishes in solutions of 8 and 12 p.p.m. of Dalapon for periods of 5 days prior to freezing. Tap water was added in the control series. Following exposure to -18° C. in the deep-freezer for periods of 4 and 8 minutes, with a 30-minute interval be-

tween freezing, the intact seedlings, contained in Petri dishes, were removed to side benches to thaw at room temperature. Approximately one hour after thawing, water was added in limited quantities to each treatment, and the moisture level was checked periodically thereafter. Recovery counts based on the average of 4 replicates of each treatment (total of 100 seedlings) were taken 5 days after freezing. The results, together with those of an experiment which duplicated the one described above as nearly as possible, are given in table 16.

Table 16. Percentage recovery of wheat seedlings germinated in various concentrations of Dalapon after exposure to -18° C. for periods of 4 and 8 minutes.

Variety	Treatment		
	Control	8 p.p.m.	12 p.p.m.
MC - first test	36	73	44
MC - second test	56	55	38

The observed increase in percentage recovery in both 8 and 12 p.p.m. treatments over the control, as indicated in the first test, is not evident in the second experiment, in which case both 8 and 12-p.p.m.-treated plants show less recovery than the comparative check treatment. As mentioned previously, subjection of intact, exposed seedlings to quick-

freezing temperatures constitutes a severe freezing test, and in many cases where damage appeared slight 2 to 3 days after freezing it tended to increase with time, often resulting in death. The evaluation of cold resistance based on apparent recovery, taken 4 to 5 days after freezing, is thus debatable under these circumstances. Moisture differences between replicates and variability within individual varieties introduce further errors. In these particular tests, considerable variability was evident in the counts on recovery of replicates of the same treatment, including the controls.

In another test with the variety MC, seeds were germinated in vermiculite with solutions of tap water, and of 8 p.p.m. of Dalapon, for 5 days at approximately 22° C. Uniform numbers of seedlings of both treatments were selected at this time and exposed, by means of the tape technique, to -15° C. in the freezer for various periods. Seedlings of both treatments were also subjected to an additional 5 days' growth at continuous low temperature (0° C.) in darkness prior to freezing. The results, based on the average of 4 replicates of each treatment, are shown in table 17.

The effect of 5 days' hardening in darkness upon both control and Dalapon-treated plants is shown by their marked increase in recovery over the unhardened material, the increase being slightly greater in hardened Dalapon-treated plants. Although both 8 p.p.m. treatments of Dalapon show

Table 17. Percentage recovery of wheat seedlings after exposure for 12 minutes at -15° C. of material with and without Dalapon and low-temperature hardening treatment.

Treatments			
Unhardened		Hardened 5 days	
Control	8 p.p.m.	Control	8 p.p.m.
4	7	79	84

slight increases in recovery over their corresponding controls, it is doubtful if these increases are significant, since the variability in percentage of recovery between replicates within treatments was quite marked.

Additional tests with wheat seedlings germinated in measured amounts of sterilized sand with concentrations of 4, 8 and 12 p.p.m. of Dalapon for periods of 72 hours, prior to freezing at -24° C. for periods of 5, 10, 15 and 20 minutes, failed to show any increase in recovery in Dalapon-treated plants compared to the control plants, which were germinated in tap water.

Limited tests, utilizing slow-freezing methods, were carried out with wheat plants treated with maleic hydrazide. Several varieties of wheat were grown to the 2 to 3-leaf stage of growth (20 days after planting) in pots in the

greenhouse at approximately 24° C. prior to initiation of hardening. Each pot contained a measured amount of well-mixed soil and sand in the proportion of 2:1, respectively. Seven days after emergence, one-half of the material was treated with maleic hydrazide at a calculated rate of 5 pounds per acre. One week following this application, when hardening was initiated, the average height of the control plants in 4 of the 5 varieties was less than the corresponding treated plants. The hardening and freezing treatment consisted of 10 days at a temperature of 0° to 1° C. in continuous light (300 W. at an average distance of 4 feet), followed by 10 days of alternating temperature in light and darkness. The temperature during the daily 8-hour light period remained at 15° C. (65° F.). During the remaining 16-hour dark period, the temperature was progressively lowered by one degree every 24 hours, from -1° to -10° C. A measured amount of water in slight excess was added to each treatment 5 and 10 days after commencement of hardening. No additional water was added until the pots were removed to the greenhouse. Two weeks after the final freezing, recovery was found to be nil in all treatments. A record of the low-temperature exposure at which injury was first apparent and when it appeared to be complete in the various varieties is shown in table 18.

Table 18. Low-temperature exposure at which freezing injury was first evident, and when injury appeared to be complete, in wheat plants of various varieties exposed periodically to successively lower air temperatures.

Variety	*Freezing temperature (°C.) at which injury: was first apparent	appeared complete
K	-7	-11
M	-7	-11
JF	-6	-10
S	-6	-10
T	-5	-9

* An absolute correction of +1° has been applied to the temperature recorder readings which were employed throughout the experiment. A further correction of at least ±0.5° C. should perhaps be applied.

A serious omission in this experiment was the failure to record minimum soil temperatures throughout the freezing periods. Since it was originally intended that the hardening treatment should cease at a minimum air temperature of -5° C., after which soil temperatures could be taken during a final more critical freezing test, soil temperatures were neglected.

It appears that the plants did not increase in cold resistance, under the methods employed, to the same extent as plants described in previous longer-term hardening treatments (3 weeks' constant low temperature at 0° to 1° C.

in continuous light). However, the hardier varieties, MC and M, although eventually killed, did not exhibit complete injury until several days after final freezing, which indicates an appreciable increase in cold resistance.

Although differences in cold resistance between maleic-hydrazide-treated plants and the comparative controls were not evident in the final analysis, there appeared to be slight differences in resistance to low temperatures prior to final freezing. The main culms of some of the chemically-treated plants appeared to be more brittle than those in the control series, and were the last to collapse prior to complete injury. Similar effects have been reported by Corns (7) with parsnips treated with other chemicals.

3. Discussion

Numerous freezing experiments with unhardened wheat varieties suggest that such tests are of little use in evaluating relative cold resistance. This becomes apparent when it is realized that cold resistance in wheat plants is acquired in varying degree at varying rates, depending upon variety and depending upon the conditions of hardening. The extensive studies of Worzella and Cutler (71) concerning the effect of weather conditions on field hardening and cold resistance in winter wheat serve to illustrate this point.

Martin (37) suggested that a rough measure of hardiness could be obtained without any previous hardening, although he noted that plants should be subjected to successively lower temperatures for more accurate measurements. The results of freezing tests with unhardened and hardened wheat varieties, presented herein, are in agreement with those of Hill and Salmon (25), who reported that the relative cold resistance of wheat varieties depends upon the degree of hardening. For this reason, freezing tests with unhardened material appear to have limited usefulness.

The results of limited freezing tests with several varieties of wheat seedlings, which were vernalized for various periods of time, indicated a rapid decline in cold resistance following completion of vernalization (at approximately 60 days). This is in general agreement with various Russian reports (4, 66). According to Murneek and Whyte et al (45), several Russian workers have found that frost resistance in winter cereals is often markedly lower in plants grown from fully vernalized seeds. Vasiljev (66) and Timofeeva (64) have reported a reduction in hardiness of winter plants after completion of vernalization. The latter worker suggested that hardening and the vernalization process occurred at the same time. Tumanov, as reported by Whyte (68), found that fully vernalized plants were incapable of further hardening.

Some investigators believe that cold resistance in vernalized plants does not begin to decrease until they are

subjected to high temperatures and long days (68). The results reported here (table 7) do not support this view. Freezing tests on material subjected to periods of vernalization longer than 80 days showed little or no recovery from exposure to -20° C. for a period of 12 minutes.

No explanation can be offered for the apparent marked increase in hardiness observed in the spring variety, T, after approximately 60 days' vernalization. Eight weeks (56 days) after initiation of vernalization, the total carbohydrates in this variety had decreased by approximately 80 per cent, and thus may have been limiting after 70 days' treatment, at which time the cold resistance of T appeared negligible.

The results of freezing tests with germinating seedlings grown in vermiculite, and hardened for periods up to 4 days prior to short periods of exposure in the freezer at temperatures of -15° to -20° C., failed to show any consistent varietal relationship with respect to known winter-hardiness. Failure to obtain a uniform relationship may have been due to insufficient periods of hardening. On the other hand, slower freezing rates at generally milder temperatures might have provided more critical results, especially where recovery from freezing was debatable under conditions of rapid freezing. Worzella (70), and Worzella and Cutler (71), found that germinating seedlings in the one-leaf or coleoptile stage of growth were quite sensitive to cold. Suneson and Peltier (63) re-

ported that young plants (4 days after emergence at initiation of hardening) were more cold-resistant than those which had emerged 25 days prior to hardening and freezing. Worzella used brief periods of hardening (15 hours at 34° F. in light), while Suneson and Peltier employed a minimum of 7 days at 33° to 39° F. in darkness.

Results of freezing tests after exposure to 7 days' hardening at constant low temperature in darkness and light (tables 8 and 9) show that it is possible to detect varietal differences in hardiness which correspond closely to known winter survival. However, the method of testing germinating seedlings by growing and freezing in vermiculite is difficult to duplicate, due mainly to moisture differences between samples. For this reason, it seems advisable to test such material in pots where moisture may be equalized more easily prior to slow freezing. Under such conditions, provision must be made for drainage of excess water applied at a given time prior to initiation of freezing.

It appears that periods of hardening of up to 15 days, at alternating high and low temperatures in light and darkness, are not too effective in promoting the development of hardiness (table 10). Several investigators, including Dexter (12), Suneson and Peltier (63), and Peltier and Kiesselbach (50), have found constant low temperature to be more effective than alternating temperatures in light and darkness in the development of hardiness in wheat plants. Dexter

employed continuous low temperature in both light and darkness and found hardening in darkness to be effective if a plentiful supply of reserve food were present in the plant. The latter authors, utilizing constant low-temperature exposure in darkness, found that increased resistance was obtained in young seedlings up to a point which apparently corresponded to endosperm exhaustion of the seeds, after which decreased resistance was observed. Their results are thus in agreement with Dexter's suggestion, noted above, that effective hardening in darkness at continuous low temperature is dependent on the supply of reserve food in the plant.

Germination, hardening for extended periods of 3 to 4 weeks, and subsequent freezing of exposed, intact seedlings in Petri dishes, does not appear to provide a satisfactory measure of varietal hardiness. The desirable features of this method of testing are more than outweighed by the higher incidence of disease and the vulnerability of the seedlings to freezing injury. Where damage is complete, the effect is evident, but where damage is lacking it is not known whether this is due to inherent cold resistance or whether the seedlings have escaped freezing injury. Partial injury, on the other hand, and subsequent recovery, as evidenced by further growth, is difficult to obtain.

Recovery counts in potted material, hardened for 3 and 6 weeks at the 2 to 3-leaf stage of growth prior to slow freezing, were in close agreement with known varietal hardiness

throughout both treatments. Intervarietal differences in cold resistance were distinct after 3 weeks' constant low-temperature treatment in continuous light, and it appears that the hardening period might be shortened considerably and still show the desired effect. In testing the relative hardiness of wheat varieties, Suneson and Peltier (63) obtained agreement with known winter survival by the use of constant low temperature (29° to 35° F.) for minimum periods of 7 days prior to freezing. The plants were germinated and grown at 60° F. for 2 days prior to hardening. Worzella (70) employed a 15-hour hardening period at 34° F. under continuous light, after growing wheat plants in the greenhouse for approximately 5 weeks. Estimated survival, after exposure to 22° F. (-5.5° C.) for 8 hours, taken 7 days after freezing, showed distinct differences between non-hardy, mid-hardy and hardy types. Whether such a limited degree of hardening will distinguish clearly smaller intervarietal differences in hardiness is debatable. In any event, the possibility of utilizing much shorter hardening periods than 3 weeks in distinguishing relative hardiness in wheat is clearly evident.

The effect of low concentrations of various growth substances, notably Dalapon, upon subsequent cold resistance of wheat seedlings appeared to be negligible. The small but significant increase in cold resistance obtained by Corns and co-workers (8, 9, 10, 44) in Dalapon-treated garden and sugar-beet seedlings was not evident in this study in repeated tests

with germinating wheat seedlings. This is in agreement with Corns (8), who found no effective increase in cold resistance in Dalapon-treated seedlings of wheat. The erratic results generally obtained where substrate was included were due in part to lack of uniformity in moisture between samples, which has been referred to previously. Intervarietal variations in degree of hardening also appeared to be less distinct with limited hardening treatment, although further testing is needed to explain discrepant results obtained with the various freezing methods.

Limited tests with maleic-hydrazide-treated plants, hardened under alternate high and low temperatures in light and darkness for extended periods, failed to distinguish differences in cold resistance when compared to the untreated controls. According to Levitt (32), Kessler and Ruhland obtained an increase in frost resistance of wheat seedlings by treatment with a growth-inhibiting substance. Further, more critical testing is needed, however, to determine the possible effect of maleic hydrazide in relation to subsequent hardiness.

III. Biochemical Tests

1. Materials and Methods

Seedlings in the one-leaf or coleoptile stage, which were grown in Petri dishes and subjected to biochemical analysis, were weighed, autoclaved for 5 minutes at 5 pounds pressure, and dried to constant weight at 65° C., as suggested by Loomis and Shull (35). The seed-pieces (remaining portion of the seed) were then carefully separated from the seedlings; the two portions were ground as finely as possible with a mortar and pestle, re-dried to constant weight at 65° C., and stored in tightly-stoppered desiccators prior to analysis.

With potted seedlings, at the 2 to 3-leaf stage of growth, the soil was washed free of the roots as carefully and quickly as possible, the seedlings were sectioned 3 mm. above the crown*, and the two portions (namely, the leaves and the crown and root portion) were weighed rapidly, autoclaved and dried to constant weight at 65° C. Following initial drying, the material was ground finely, re-dried at the same temperature, and stored in desiccators.

Total nitrogen was determined by the micro-Kjeldahl method, as outlined by the A.O.A.C. (3) except that

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* Where the crown node was undeveloped, the seedlings were sectioned 15 mm. above the point of attachment of the seed-piece.

a one-hour digestion period was used. After 30 minutes' digestion, the flasks were cooled and 3 drops of Superoxol were added. Further digestion for 20 minutes, followed by cooling, addition of Superoxol and a final 10-minute digestion period, constituted the procedure prior to distillation.

Soluble nitrogen was extracted from 100 mgm. of material in 20 ml. of distilled water by continuous shaking for 16 hours and filtering to remove the residue. Two ml. of Galston's and Dalberg's (17) digestion mixture were added to 2 ml. of the extract, and a one-hour digestion, as described above, was carried out prior to final distillation.

Soluble protein nitrogen was determined by precipitation of a 4-ml. aliquot of the extract with an equal volume of 1 M trichloroacetic acid in a Lusteroid centrifuge tube. After 16 hours' refrigeration, the tubes were centrifuged for 10 minutes at 3500 g. and the supernatant liquid carefully decanted. The precipitate was transferred to a 35-ml. digestion flask with the aid of 2 ml. of digestion mixture and several washings of distilled water (2 to 3 ml.). The samples were then digested and distilled, as described above. The method of determining soluble protein nitrogen was essentially the same as that described by Galston and Dalberg (17).

Total sugars were removed by extracting 50 mgm. of sample with 80% ethanol for 6 hours in a Soxhlet apparatus.

Following evaporation of the alcohol, the samples were cleared with saturated neutral lead acetate and the excess lead removed with saturated disodium phosphate. Small amounts of animal charcoal were added and the mixture allowed to stand, with frequent shaking, for 30 minutes before filtering in a Buchner funnel containing a thin layer of talc on moistened filter paper. Ten ml. of HCl (specific gravity, 1.1029) were then added to each flask and the samples set aside for 24 hours at room temperature to permit hydrolysis to monosaccharides. Following hydrolysis, the samples were neutralized with sodium carbonate and brought up to volume. Total sugars were determined by the method of Hassid (23, 24) and the results expressed as per cent glucose.

Starch was determined according to the method of Loomis and Shull (35), except that one-tenth quantities were used. After drying the sugar-free material, samples of approximately 25 to 30 mgm. were obtained for starch analysis. The results were also expressed as per cent glucose.

2. Results

(1) Germinative stage

(a) Nitrogen analysis

Nitrogen determinations (including, total N, soluble N and soluble protein N) were carried out on wheat seedlings, which were exposed in Petri dishes for 4 and 8 weeks

at a constant temperature of 0° to 1° C., in darkness and light (150 W. at a distance of approximately 4 feet), after 2 days initial germination at room temperature. The filter paper, contained in each Petri dish, was kept as uniformly moist as possible by the regular addition of demineralized water. The percentage moisture of the varieties after completion of the various hardening treatments is shown in table 19.

Table 19. Percentage moisture content of seedlings of 5 wheat varieties after 4 and 8 weeks' exposure to 0° to 1° C. in continuous light and darkness (dry weight basis at 65° C.).

Variety	Hardening conditions			
	Light		Dark	
	4 weeks	8 weeks	4 weeks	8 weeks
MC	60	70	71	83
M	65	74	76	84
JF	63	72	74	82
S	64	73	70	87
T	63	72	79	86

Marked differences in moisture percentage are clearly evident between 4 and 8-week treatments in light and in darkness, being approximately 10 per cent higher in both cases after

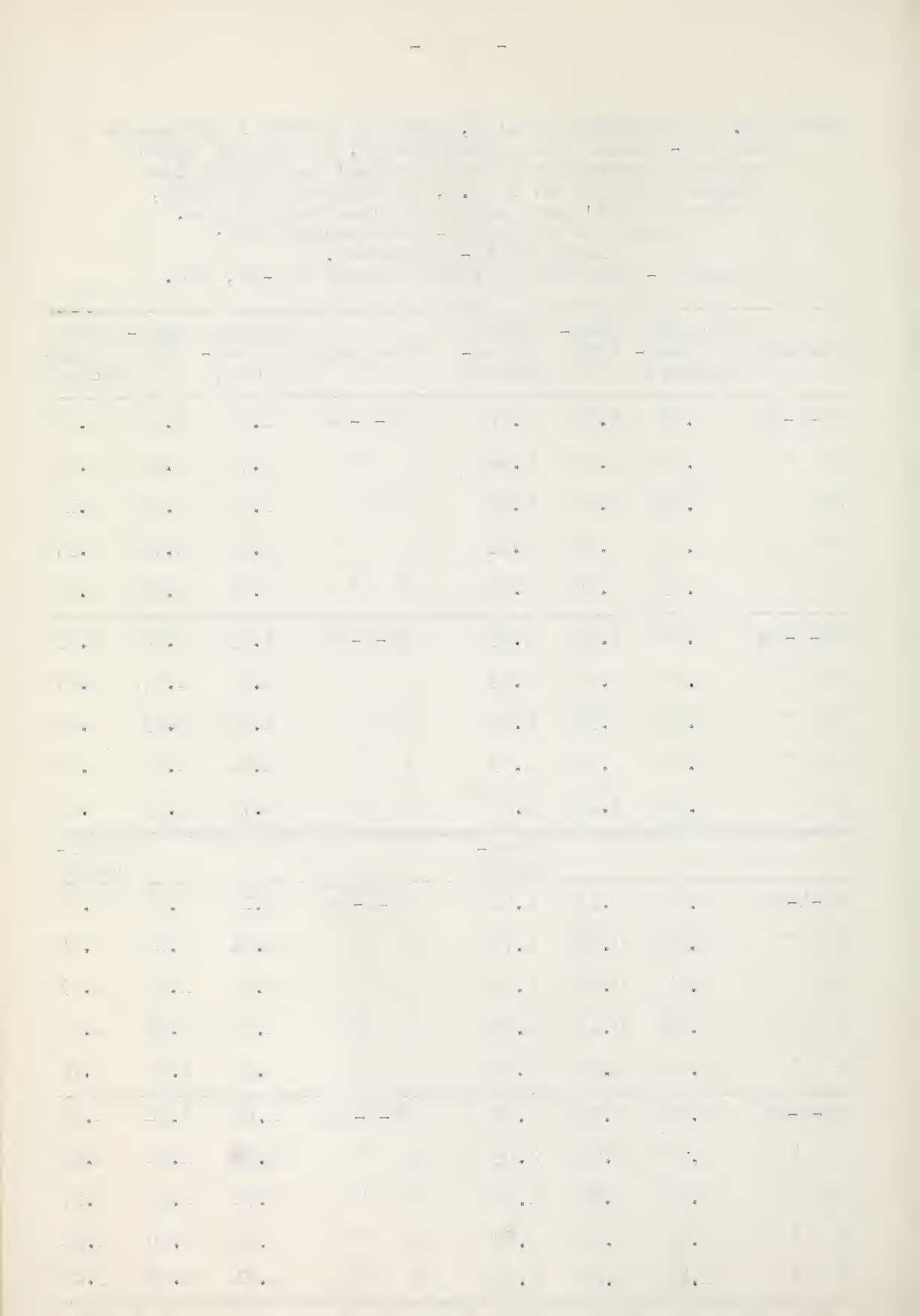
an additional 4 weeks' low-temperature exposure. Moisture differences between "4-week-light" and "8-week dark" exposures are still greater, being approximately 20 per cent or more higher in the latter treatments. Because of the incidence of diseased seedlings and the fact that material was limiting, it was considered impractical to compare the various treatments on total dry weight, based on a uniform number of plants. After nitrogen analyses were completed, however, the percentage N in the various fractions (total N, soluble N, etc.) was re-calculated according to the proportion of total residual seed-piece weight or total weight of seedling portion to total dry weight of the original material. For comparative purposes, total N determinations of the residual seed-piece material and seedlings, which were separated after initial drying, will be shown before and after correction, on the basis noted above, in table 20.

Further comparisons may be made from the results of total and soluble N determinations on ground seed samples of the various varieties, dried to constant weight at 65° C., after autoclaving. The results are listed below in per cent:

Variety	Sample	Total N	Soluble N
MC	whole ground seed	1.84	0.35
M	" " "	1.74	0.33
JF	" " "	1.68	0.39
S	" " "	1.55	0.36
T	" " "	2.23	0.40

Table 20. Percentage total N, shown as residual or reserve N (in seed-pieces) and as seedling N, of various wheat varieties exposed to 4 and 8 weeks' continuous low temperature (0° to 1° C.), in darkness and light, after 2 days' germination at room temperature. (Upper half of table - uncorrected data; lower half - corrected.) Legend - hardened in light 4 weeks = l-4w, etc.

Treatment	Reserve N (seed-pieces)	Seed-ling N	Total (uncor-rected)	Treatment	Reserve N (seed-pieces)	Seed-ling N	Total (uncor-rected)
MC-l-4w	1.72	2.58	4.30	MC-l-8w	1.95	2.43	4.38
M "	1.70	2.34	4.04	M "	1.74	2.74	4.48
JF "	1.68	2.60	4.28	JF "	1.59	2.60	4.19
S "	1.53	2.70	4.23	S "	1.59	2.78	4.37
T "	2.23	2.99	5.22	T "	2.54	3.42	5.96
MC-d-4w	1.70	2.85	4.55	MC-d-8w	2.45	3.38	5.83
M "	1.75	2.68	4.43	M "	1.90	2.77	4.67
JF "	1.60	3.36	4.96	JF "	1.86	2.63	4.49
S "	1.57	3.16	4.73	S "	2.00	2.78	4.78
T "	2.36	3.10	5.46	T "	2.78	4.05	6.83
			(cor-rected)				(cor-rected)
MC-l-4w	1.54	0.17	1.71	MC-l-8w	1.10	0.84	1.94
M "	1.34	0.42	1.76	M "	0.91	1.18	2.09
JF "	1.44	0.26	1.70	JF "	0.93	1.00	1.93
S "	1.38	0.18	1.56	S "	1.04	0.86	1.90
T "	1.89	0.36	2.25	T "	1.52	1.22	2.74
MC-d-4w	1.25	0.58	1.83	MC-d-8w	1.06	1.82	2.88
M "	1.08	0.95	2.03	M "	0.56	1.52	2.08
JF "	1.15	0.84	1.99	JF "	0.72	1.45	2.17
S "	1.20	0.64	1.84	S "	0.73	1.69	2.42
T "	1.45	1.09	2.54	T "	1.21	2.08	3.29



Total soluble N determinations are presented in table 21 in the same manner (uncorrected and corrected data).

Table 21. Percentage total soluble N, shown as reserve N (residual seed-piece material) and as seedling N, of various wheat varieties exposed to 4 and 8 weeks' continuous low temperature (0° to 1° C.), in darkness and light, after 2 days' germination at room temperature. (Upper portion of table - uncorrected data; lower portion - corrected data.)

Treatment	Reserve N (seed-pieces)	Seed-ling N	Total (uncor-rected)	Treatment	Reserve N (seed-pieces)	Seed-ling N	Total (uncor-rected)
MC-1-4w	0.35	0.69	1.04	MC-1-8w	0.56	0.92	1.48
M "	0.67	0.79	1.46	M "	0.68	0.98	1.66
JF "	0.58	0.63	1.21	JF "	0.77	1.06	1.83
S "	0.37	0.67	1.04	S "	0.56	0.96	1.52
T "	0.82	0.82	1.64	T "	1.04	1.45	2.49
MC-d-4w	0.48	0.92	1.40	MC-d-8w	0.99	1.48	2.47
M "	0.64	0.94	1.58	M "	0.72	1.14	1.86
JF "	0.42	1.19	1.61	JF "	0.54	1.17	1.71
S "	0.57	1.03	1.60	S "	0.57	0.92	1.49
T "	0.72	1.24	1.96	T "	1.10	2.02	3.12
		(corrected)					(corrected)
MC-1-4w	0.31	0.04	0.35	MC-1-8w	0.32	0.32	0.64
M "	0.63	0.14	0.77	M "	0.35	0.42	0.77
JF "	0.50	0.06	0.56	JF "	0.45	0.41	0.86
S "	0.33	0.04	0.37	S "	0.37	0.30	0.67
T "	0.69	0.10	0.79	T "	0.62	0.52	1.14
MC-d-4w	0.35	0.19	0.54	MC-d-8w	0.43	0.80	1.23
M "	0.39	0.33	0.72	M "	0.21	0.62	0.83
JF "	0.30	0.30	0.60	JF "	0.21	0.65	0.86
S "	0.44	0.21	0.65	S "	0.21	0.56	0.77
T "	0.44	0.44	0.88	T "	0.48	1.04	1.52

Although increases in both total and soluble N between "4 and 8-week-treated" seedlings in light and darkness are generally evident in uncorrected data, they are more distinct when shown on a proportionate basis, as indicated in the corrected results. This is shown more clearly if we rearrange the corrected data in three of the varieties and include insoluble N (obtained by subtraction of soluble N from total N) as shown in table 22.

As the total reserve N is depleted in the seed, corresponding increases in all nitrogen fractions (total, insoluble and soluble) are evident in the seedlings of each variety. However, because of the additional influence of light, comparisons are difficult within light and dark treatments. Such comparisons are shown below, where the percentage increase in seedling N of the "8-week-treated" over the "4-week-treated" seedlings is shown under conditions of light and dark in table 23.

Although the hardiest variety, MC, shows the greatest increases in N within the "dark-treated" varieties, the hardiness reltionship is not constant in either dark or light treatments. The tender spring variety, T, shows more increase in per cent N than JF in darkness, and the greatest increase in all fractions in continuous light.

Soluble-protein-nitrogen determinations, which are not shown, were also carried out on the separated portions of the 5 varieties exposed to the various hardening treatments.

Table 22. Nitrogen content of seedlings of 3 varieties of wheat after exposure to 4 and 8 weeks' continuous low temperature (0° to 1° C.) in continuous light and darkness. (Nitrogen shown in per cent.)

Variety and treatment	Reserve N (seed-pieces)			Seedling N			Totals (seed-piece + seedling)			TOTAL N
	Total N	Insol. N	Soluble N	Total N	Insol. N	Soluble N	Total N	Insol. N	Soluble N	Intact seed sample
MC-1-4w	1.54	1.23	0.31	0.17	0.13	0.04	1.71	1.36	0.35	
MC-d-4w	1.25	0.90	0.35	0.58	0.39	0.19	1.83	1.29	0.54	un-treated control
MC-1-8w	1.10	0.78	0.32	0.84	0.52	0.32	1.94	1.30	0.64	1.84
MC-d-8w	1.06	0.63	0.43	1.82	1.02	0.80	2.88	1.65	1.23	
JF-1-4w	1.44	0.94	0.50	0.26	0.20	0.06	1.70	1.14	0.56	
JF-d-4w	1.15	0.85	0.30	0.84	0.54	0.30	1.99	1.39	0.60	un-treated control
JF-1-8w	0.93	0.48	0.45	1.00	0.59	0.41	1.93	1.07	0.86	1.68
JF-d-8w	0.72	0.51	0.21	1.45	0.80	0.65	2.17	1.31	0.86	
T-1-4w	1.89	1.20	0.69	0.36	0.26	0.10	2.25	1.46	0.79	
T-d-4w	1.45	1.01	0.44	1.09	0.65	0.44	2.54	1.66	0.88	un-treated control
T-1-8w	1.52	0.90	0.62	1.22	0.70	0.52	2.74	1.60	1.14	2.23
T-d-8w	1.21	0.73	0.48	2.08	1.04	1.04	3.29	1.77	1.52	

Table 23. Percentage increase in seedling N of "8-week-hardened" as compared to "4-week-hardened" seedlings exposed to continuous low temperature in darkness and light.

Variety	% increase in N of seedlings exposed to an additional 4 weeks in:					
	darkness			light		
	Total N	Insoluble N	Soluble N	Total N	Insoluble N	Soluble N
MC	1.24	0.63	0.61	0.67	0.39	0.28
JF	0.61	0.26	0.35	0.74	0.39	0.35
T	0.99	0.39	0.60	0.86	0.44	0.42

Since only [redacted] traces of soluble protein N were discovered in the various samples, regardless of hardening treatment, the results are omitted.

(b) Sugar and starch analysis

In an attempt to explain some of the observed differences in the nitrogen fractions under the various hardening conditions, it was decided to analyze the treatments hardened for 8 weeks in light and darkness for total carbohydrate content. Total sugars and total starch determinations (both expressed as per cent glucose) were carried out on the reserve material (seed-pieces) and the seedling portion of 3 varieties of germinating wheat seedlings. The results are shown in table 24.

Table 24. Total carbohydrates, shown as total sugar and starch (expressed as per cent glucose), present in reserve and seedling material of 3 wheat varieties exposed to 8 weeks' continuous low temperature in light and darkness. (Data corrected as for N determinations.)

Variety and treatment	Total sugars			Total starch			Total carbohydrates
	Reserve (seed-pieces	Seed-lings	Total	Reserve (seed-pieces)	Seed-lings	Total	(Grand total)
MC-ground seed (control)			1.1			84.3	85.4
MC-l-8w	11.4	3.9	15.3	23.6	3.0	26.6	41.9
MC-d-8w	7.5	6.3	13.8	13.0	3.9	16.9	30.7
JF-ground seed (control)			1.7			85.7	87.4
JF-l-8w	19.7	7.1	26.8	33.4	6.5	39.9	66.7
JF-d-8w	10.0	10.3	20.3	10.6	12.2	22.8	43.1
T-ground seed (control)			1.4			68.0	69.4
T-l-8w	5.8	4.5	10.3	15.8	3.6	19.4	29.7
T-d-8w	3.6	3.2	6.8	6.0	2.0	8.0	14.8

Analysis of the control material shows that there is little carbohydrate present in the form of sugar in dry seeds of both winter and spring varieties. The amount of stored starch, however, is much higher in the two winter varieties

than in T, being at least 16 per cent greater in both MC and JF in dry seeds. In growing material, the "8-week-treatment" of JF maintained in light is seen to have the greatest food reserve in the form of total sugar and starch. While total carbohydrates are not limiting in any treatment, the lowest available carbohydrate supply is evident in T after 8 weeks' continuous low temperature in darkness. The decrease in percentage total carbohydrate supply is shown in table 25 for the various treatments.

Table 25. Decrease in percentage total carbohydrates of wheat seedlings after 8 weeks' low-temperature exposure in light and darkness.

Variety	Decrease in percentage total carbohydrates after 8 weeks in:		Difference presumably due to photosynthesis
	darkness	light	
MC	54.7	43.5	12.2
JF	44.3	20.7	23.6
T	54.6	39.7	14.9

Under the conditions employed, it appears that, of the 3 varieties, JF is best able to conserve carbohydrates in both darkness and light. In the latter instance, this is apparently due to more efficient photosynthetic activity. Comparison of the hardy winter variety, MC, and the tender spring variety, T, shows little difference in this respect.

(2) Seedling stage (2 to 3-leaf stage of growth)

(a) Nitrogen analysis

Total water-soluble N and water-soluble protein N determinations were also carried out on the leaf, and crown-and-root portion of seedlings exposed to 3 and 6 weeks' hardening at 0° to 1° C. in continuous light (300 W. at an average distance of 4 feet) in the 2 to 3-leaf stage of growth (20 days after planting in the greenhouse). The average heights of the seedlings at initiation and at completion of hardening were shown previously in table 12. The method of sectioning the material has also been referred to. The results of these determinations, together with percentage moisture (dry-weight basis at 65° C.) for the various treatments, are shown in table 26, along with the data for unhardened controls.

In the hardy variety, MC, total soluble N varies inversely with increased periods of hardening. The remaining varieties show a marked decrease in soluble N after 3 weeks' low-temperature exposure, after which a gradual increase is apparent. The rapid decrease in soluble N and subsequent increase after 6 weeks' hardening is especially marked in the spring variety, T.

On the other hand, the limited amounts of water-soluble protein N obtained in the unhardened material become even more limiting with increased periods of hardening, being detected in only small amounts after 3 and 6 weeks' exposure to continuous low temperature.

Table 26. Percentage water-soluble N and water-soluble protein N, based on leaf and crown-and-root portions of wheat seedlings after exposure to 3 and 6 weeks' low temperature (0° to 1° C.) in continuous light, of wheat seedlings in the 2 to 3-leaf stage of growth.

Variety and treatment	% moisture at 65° C.	Water-soluble N			Water-soluble protein N		
		Leaves	Crowns and roots	Total	Leaves	Crowns and roots	Total
MC-control	95.1	1.17	0.29	1.46	0.15	0.03	0.18
M "	94.7	1.03	0.28	1.31	0.10	0.02	0.12
JF "	93.3	1.34	0.33	1.67	0.09	0.02	0.11
S "	94.9	0.87	0.33	1.20	0.05	0.02	0.07
T "	93.7	1.56	0.22	1.78	0.06	0.02	0.08
MC-1-3w	89.6	0.57	0.15	0.72	0.05	trace	0.05
M "	89.7	0.61	0.13	0.74	0.10	trace	0.10
JF "	89.5	0.52	0.20	0.72	0.03	0.02	0.05
S "	90.7	0.58	0.20	0.78	0.03	0.02	0.05
T "	91.9	0.49	0.17	0.66	0.04	trace	0.04
MC-1-6w	85.4	0.52	0.14	0.66	0.01	0.01	0.02
M "	88.1	0.64	0.18	0.82	0.01	0.01	0.02
JF "	87.0	0.65	0.14	0.79	0.04	0.01	0.05
S "	90.6	0.68	0.18	0.86	0.01	0.01	0.02
T "	91.6	0.99	0.16	1.15	0.03	0.01	0.04

3. Discussion

There appears to be no constant relationship between varietal hardiness and nitrogen content of wheat seedlings as determined under the conditions of these studies. The observed increases in both total and soluble N in germinating wheat seedlings were not consistent with the known order of hardiness throughout the varieties tested, regardless of method of hardening. Similar results are reported by Newton (46), Dexter (14), Levitt (32), and others.

There appeared to be no correlation between changes in water-soluble protein N and hardiness of the various varieties. This is in contrast to work by Siminovitch and Briggs (57) with live bark of Robinia spp., by Smith and Hodgson (5) with alfalfa, and by Hodgson and Bula (26) with sweet clover. The lack of correlation might be attributed to insignificant amounts of soluble protein N, or insufficient hardening since it appears to exist only in well-hardened material. The small size of sample used in testing for soluble protein N may have limited its detection, since it appears to be present in minute amounts in wheat plants. On the other hand, this protein fraction may only be evident in appreciable amounts in hardier plants than wheat. Bula, Smith and Hodgson (5) reported that increases in water-soluble protein did not always coincide with sharp increases in cold resistance of alfalfa.

In attempting to relate carbohydrate losses with observed changes in nitrogen, a direct effect upon growth might

be implied. It should be emphasized, however, that the germinating seedlings exposed to 4 and 8 weeks' low temperature in darkness and light had only one source of N, namely, that stored in the seed. Thus the seedlings which show the greatest loss in carbohydrates might be expected to show the greatest growth (up to the point of endosperm exhaustion), which, in turn, is reflected in greater amounts of total seedling N. Comparison of tables 23 and 25 shows such a varietal trend in seedlings exposed to 8 weeks' darkness. The variety, JF, shows the least increase in total seedling N (table 23) and the least decrease in total carbohydrates (table 25). MC and T are in general agreement with respect to total N, each having used the same amount of carbohydrate material. Similar comparisons are obscured in light, however, due to the additional effect of photosynthesis.

The clearly observable differences in amount of growth (in length) of seedlings exposed to the various hardening treatments are shown in order of increasing growth rate in table 22, gradually increasing in the "4-week-light" to "8-week-dark" treatments. Similar trends are also evident on a percentage moisture-content basis for the various treatments (table 19).

Since the seedlings germinated in darkness at constant low temperature for 8 weeks are essentially vernalized, the results should be comparable, in part, to the nitrogen analyses of Konovalov, as reported by Murneek and Whyte et al

(45). Konovalov found that with prevention of vernalization the breakdown of the proteins extended to the end products, whereas during vernalization the proteins retained their form but became more readily soluble. He concluded that nitrogenous substances appeared to be resynthesized during vernalization, and regarded this as a distinctive feature of the vernalization process. Although the statement appears ambiguous, the inference of resynthesis seems debatable. Further criticism is, perhaps, unjustified without clarity of interpretation.

It will be observed in table 22 that the sums of the reserve and seedling N correspond quite well with the total N of the untreated controls in all but the longer dark treatments (the differences are a little more pronounced in both 8-week treatments of T). A proportionately greater loss of carbohydrate as compared to N from the seed would tend to show higher values for reserve N than was actually the case in individual samples. The discrepancies in nitrogen totals may thus be more apparent than real, as observed in the vernalized material (8 weeks low-temperature germination in darkness) where carbohydrate depletion is the greatest. This view is contrary to Konovalov's suggestion of resynthesis of nitrogenous substances in vernalized material.

In the material studied, a considerable breakdown in protein was apparent in the reserve material of the seed.

This was accompanied by a marked increase in the seedlings of all nitrogen fractions except soluble protein N. The observed increases in total seedling N were at the expense of N stored in the seed, since no other source of N was supplied throughout the low-temperature treatment.

SUMMARY AND CONCLUSIONS

The relationship between length of vernalization period and known hardiness of several wheat varieties has been studied. There appeared to be no correlation between length of time required to complete vernalization and known winter-hardiness of the various varieties. The reasons for the observed irregularities in time of heading between the varieties have been discussed.

Unhardened material in both the germinative and the 2 to 4-leaf stages of growth was tested by slow and quick-freezing techniques. It was found to provide an unreliable index of winter-hardiness in the varieties studied.

Wheat seedlings, vernalized for periods ranging up to 80 days, gradually increased in cold resistance in all varieties up to the sixtieth day, which corresponded to the point of complete vernalization. Beyond this point there was a marked decline in cold tolerance.

Subjection of free, intact wheat seedlings in the germinative stage to constant low temperature for periods varying from 2 to 14 days, prior to freezing, indicated: (a) that it was impracticable to assess freezing injury in such completely exposed material; and (b) that freezing tests on germinating seedlings grown in vermiculite and hardened

for periods of 7 and 14 days in continuous light gave a fairly satisfactory index of hardiness, while shorter hardening periods of up to 3 days were less reliable.

Longer-term hardening treatments at controlled low temperatures and continuous illumination, for periods of 3 to 6 weeks, applied during the 2 to 4-leaf stage to seedlings grown in pots and finally subjected to slow-freezing techniques, gave a satisfactory hardiness rating in comparison to winter-survivial data. Such experiments, therefore, appeared to have merit in the development of a basis for testing cold resistance of winter wheat varieties.

Pre-and post-germinative application of various chemicals, in particular Dalapon (sodium 2,2-dichloropropionate) and maleic hydrazide, gave erratic results, which failed to indicate any consistent increase in cold resistance attributable to the applied chemicals.

Biochemical analyses of the material, which had been subjected to the different hardening conditions, included analysis for several nitrogen fractions, as well as sugar and starch determinations on a portion of the material. The implications of the observed results with vernalized material have been discussed briefly with reference to previous work and an alternative explanation offered.

Seedlings analyzed in the 2 to 3-leaf stage of growth showed no increase in soluble protein nitrogen with

increasing periods of cold treatment under continuous illumination. In this respect, the situation in winter wheats was contrary to that reported in certain work with trees and legumes.

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